Neuroprotection and ageing in sensory neurons

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Abstract

Cardiotrophin-1 (CT-1) and Urocortin (UCN) are peptides which have previously been shown to have protective effects in cardiac myocytes and which induce heat shock protein expression. CT-1 is a member of the interleukin-6 family of cytokines. UCN is a peptide expressed in the brain that binds to the corticotrophin releasing hormone receptors. In this investigation the ability of CT-1 and UCN to protect cells against a lethal stress was tested in neonatal and adult sensory neurons. CT-1 has a protective effect against hypoxic ischaemia in neonatal sensory neurons; in contrast although CT-1 induces the expression of HSP 70 in adult sensory neurons it does not have a protective effect. Results from studies using the kinase inhibitors PD 98059, SB 203580 and LY 294002 suggest that CT-1 is activating the P42/44 MAPK pathway in neonatal sensory neurons. The western blots also reveal a higher level of phosphorylated P42 MAPK in the adult sensory neurons than in the neonatal sensory neurons suggesting that CT-1 does not have a protective effect in the adult neurons because this pathway is already activated. Surprisingly, UCN does not have a protective effect in sensory neurons. In the neonatal cells this could be explained by a lack of receptor expression since RT-PCR revealed an absence of mRNA for both receptors 1 and 2, however; in the adult neurons mRNA was present for both receptors. In conclusion CT-1 but not UCN has an age dependent protective effect in sensory neurons.

CT-1 is not the only treatment known to have a protective effect that is lost with age, for instance ischaemic preconditioning is protective in young hearts but not in senescent hearts. The induction of heat shock proteins by mild stress is protective against further stress in young animals but the induction of HSPs is impaired with age. Investigations into the possible therapeutic benefit of increasing heat shock protein expression have previously been performed but this work was carried out using young animals and cells from young animals. This investigation therefore progressed to explore whether increasing heat shock protein expression in neurons from aged animals could protect them from lethal stresses.

An HSV-1 based viral vector was used to individually overexpress three members of the heat shock family, HSP 27, HSP 70 and HSP 56, in sensory neurons of aged and neonatal Sprague Dawley rats *in vitro.* Transcription of the HSP genes in this vector is controlled by a CMV promoter, producing high levels of protein expression. The protective effect of the three proteins against heat shock and hypoxic ischaemia was tested in sensory neurons from aged and neonatal rats. As has been previously shown, in neonatal sensory neurons HSP 27 and HSP 70 protect against cell death due to heat shock. Encouragingly, both HSP 27 and HSP 70 protect the sensory neurons of aged animals against heat shock, although only HSP 27 gave a significant level of protection against hypoxic ischaemia in aged sensory neurons. In conclusion, it is possible to protect neuronal cells of aged animals against stress if the levels of heat shock proteins can be restored.

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Abbreviations

ACTH	Adrenocorticotrophic hormone
AHIF	Ascites HSP 72-inducing factor
AIF	Apoptosis inducing factor
AraC	Cytosine β-D arabionofuranoside
BDNF	Brain derived neurotrophic factor
Bimoclomol	N-2-hydroxy-3-(1-piperidinyl)propoxy]-3-pyridine-carboxyimidoyl chloride maleate
CLC	Cardiotrophin-1 like cytokine
CMV-IE	Cytomegalovirus immediate early promoter
CNS	Central nervous system
CNTF	Ciliary neuron trophic factor
CNTFR	Ciliary neuron trophic factor receptor
Cresyl Violet Acetate	9-Amino-5-imino-5H-benzo(a)phenoxazine acetate salt
CRF	Corticotropin releasing factor
CRFR	Corticotropin releasing factor receptor
CT-1	Cardiotrophin – 1
CT-1R	Cardiotrophin-1 receptor
ddH_20	Double distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DRG	Dorsal root ganglion
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ERK	Extracellular regulated kinase
EW	Edwinger-westphal nucleus
FCS	Foetal calf serum
FGM	Full growth medium
G418	Geneticin
GFP	Green fluorescent protein
Gp130/190	Glycoprotein 130/190

Grp78/94	Glucose regulated protein 78/94
HBSS	Hank's balanced salt solution
HMBA	Hexamethylene bisacetamide
HPA	Hypothalamic pituitary adrenal axis
HRP	Horseradish peroxidase
HSBP1	Heat shock binding protein 1
HSC	Heat shock cognate
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
HSV	Herpes Simplex Virus type 1
IGF-1	Insulin like growth factor – 1
IL-6/11	Interleukin-6/11
IL-6R	Interleukin-6 receptor
KDa	KiloDalton
Lamp2a	Lysosome associated protein 2a
LAT	Latency associated transcript
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
LSO	Lateral superior olivary nucleus
LY 294002	2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride
MAPK	Mitogen-activated protein kinase
Metrizamide	2-(3-Acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-Glucose
MOI	Multiplicity of infection
NGF	Nerve growth factor
NT-3/4	Neurotrophin-3/4
OSM	Oncostatin M
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered solution
PD 98059	2'-Amino-3'methoxyflavone
pfu	Plaque forming units

PI-3-kinase	Phosphotidylinositol-3-kinase
rpm	Revolutions per minute
SB 203580	4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SRP	Signal recognition particle
STAT	Signal transduction and activators of transcription
TEMED	NNNN-tetraethylethalinediamine
TPR	Tetratricopeptide repeat
TUNEL	Terminal deoxynucleotidytransferase-mediated dUTP nick end labeling
Tween 20	Polyoxyethylene-sorbitan monolaurate 20
UCN	Urocortin
UCN-ir	Urocortin immunoreactivity

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Chapter 1: Introduction

1.1 Sensory neurons

1.1.1 Sensory neurons described

Dorsal root ganglia sensory neurons transmit information from a variety of receptors to the central nervous system (CNS). Sensory neurons are very heterogenous and can be categorized in a number of different ways. They can be divided into sensory modalities, morphological type or by the expression of cytochemical markers.

Morphologically, sensory neurons can be divided into two categories, large light neurons and small dark neurons, these are also referred to as L and SD or A and B respectively. Although light and small dark are most commonly used it is misleading to call the light neurons large because although the mean cell size for the light neurons is larger there is a normal distribution of cell sizes within the light group that extends over the full range of cell body sizes. The small dark neurons also have a statistically normal distribution but the range is confined to the lower end of the distribution of cell sizes (Lawson, 1979).

Light neurons have uneven staining with dark clumps of Nissl substance interspersed with areas of the cell containing microtubules and neurofilament that do not stain. Nissl substance is an aggregation of ribosomes and rough endoplasmic reticulum. Small dark neurons stain more darkly and more evenly because they contain fewer neurofilaments and have more organelles (Duce and Keen, 1977).

The small dark neurons lack the 200KDa phosphorylated neurofilament protein and have largely unmyelinated C fibres. Electron microscopic analysis of dorsal root ganglia (DRG) indicates that 70 – 75% of sensory neurons that enter the spinal cord are C fibres. Light neurons which express the 200KDa neurofilament protein have myelinated A type fibres (Hunt et al., 1992). Nerve fibres are divided into Aα, Aβ, Aδ and C which are grouped by conduction velocity, fastest to slowest, and broadly reflect the diameter

of the nerve fibre, rather than reflecting the sensory modalitity. These groups have also been called I, II, III and IV (Gasser and Erlanger, 1927; Erlanger and Gasser, 1930; Lloyd and Chang, 1948; Gasser, 1950; Gasser, 1955; Perl, 1992).

In terms of modality there are sensory neurons that transmit information from many different types of receptors including muscle spindles, Golgi tendon organs, hair follicle receptors, pacinian corpuscles, Krause endings, mechanoreceptors, cool receptors and nociceptors (Carr and Nagy, 1993).

There are a large number of biochemically defined populations of sensory neurons and many of them overlap. Biochemical markers used to define populations of neurons include: peptides such as tachykinins (Substance P, neurokinin A and neuropeptide K), Calcitonin gene related peptide, somatostatin, vasoactive intestinal polypeptide, galanin, bombesin, cholecystokinin, arginine vasopressin, oxytocin, serotonin and the opiod peptides pro-dynorphin and leu-enkephalin; enzymes such as acetylcholinesterase, acid phosphatases, adenosine deaminase, tyrosine hydroxylase, carbonic anhydrase and protein kinase C; as well as neuromodulin; calbindin; parvalbumin; glutamate; the NGF receptor and the cholera toxin B subunit (Lawson, 1992).

Although it is beyond the scope of this thesis to review the various markers in detail it is clear that sensory neurons are a very diverse population of cells with many different functional and biochemical characteristics. They provide a robust and practical neuronal model because they are diverse, can be enriched to provide 90% pure neuronal cultures and can be maintained in culture for a period of weeks.

1.1.2 Neurotrophins

Different groups of sensory neurons are also dependent on different trophic factors for their survival. There are four neurotrophins in mammals that affect the survival of sensory neurons, and two neurotrophin receptor systems. The neurotrophins can bind to the p75NTR receptor or the Trk receptors A, B or C on the cell surface. Nerve growth factor (NGF) binds to TrkA, BDNF binds to Trk B as does NT4, and NT-3 binds to Trk C, all four bind to p75NTR (Chao, 2003). There is also evidence that NT-3 supports the

survival of Trk A and Trk B expressing neurons, and therefore may bind to the Trk A and B receptors as well (Farinas et al., 1998; Huang et al., 1999). Neurotrophin signalling is mediated through phospholipase C, Ras, ERK, PI-3 Kinase, NF-κB and Jun (Huang and Reichardt, 2001). The neurotrophins have many important roles not just in neuronal survival but in neuronal development and function as well. Target organs and regions through which sensory neurons grow to reach their targets release neurotrophins, neurons themselves can produce neurotrophins and non-neuronal cells produce neurotrophins after nerve injury (Korsching, 1993).

NT-3 is essential for the survival of some embryonic sensory neurons (Rosenthal et al., 1990; Maisonpierre et al., 1990; Farinas et al., 1996). BDNF supports the survival of some sensory neurons including those innervating the vascular system and correct temporal and spatial expression of BDNF is required for the correct development of gustatory neurons (Lindsay et al., 1985; Davies et al., 1986; Brady et al., 1999; Ringstedt et al., 1999). NGF supports the survival of developing sympathetic and sensory neurons and is expressed by the targets of the neurons (Johnson, Jr. et al., 1980; Heumann et al., 1984; Korsching and Thoenen, 1985). Anti-NGF antibodies cause the loss of 90% of the unmyelinated fibres in the dorsal root sensory neurons of embryonic rats (Goedert et al., 1984). Neuronal requirements for neurotrophins have been looked at in knockout mice: there is a 70% loss of DRG sensory neurons in the NGF knockout and a corresponding 70-90% loss in the Trk A knockout, a 35% loss in the BDNF knockout and a similar 30% loss in the Trk B knockout, a 60% loss in the NT-3 knockout, and a 20% loss in the Trk C knockout, showing that some sensory neurons require the presence of more than one trophic factor to survive (Huang and Reichardt, 2001).

In adult rats the loss of target derived NGF when sensory neurons are axotomized causes the loss of 15-40% of the cells (Johnson, Jr. and Yip, 1985; Arvidsson et al., 1986; Himes and Tessler, 1989), however, *in vitro* adult sensory neurons have no absolute requirements for neurotrophic factors (Lindsay, 1988). In neonatal sensory neurons NGF improves the survival of neonatal sensory neurons by about 45% (Mulderry, 1994).

Loss of sensory neurons with age is quite limited but ageing is associated in sensory neurons with degenerative events such as cell body atrophy, axon atrophy and demyelination as well as phenotypic switches and remodeling of sensory innervation, these events result in sensorimotor disturbances in the elderly (Bergman and Ulfhake, 1998; Ulfhak et al., 2002). Although the size distribution of dorsal root ganglia sensory neurons is maintained Trk receptor expression is downregulated in response to the loss of target derived ligands and this appears to play a key role in the age associated sensory disturbances (Ming et al., 1999; Bergman et al., 1999; Ulfhak et al., 2002).

1.2 Heat shock proteins

The description of puffing in *Drosophila* salivary gland chromosomes in response to heat and other treatments including anaerobiosis (Ritossa, 1962; Ritossa, 1964; Ritossa and Vonborstel, 1964) paved the way for the discovery that heat shock caused the preferential expression of a particular set of genes, a set of genes now known as the heat shock proteins (HSPs)(Tissieres et al., 1974). The literature regarding heat shock proteins and the heat shock response is now too vast to be reviewed, even briefly, in its entirety, therefore a brief general introduction to the heat shock proteins will be followed by an introduction to the particular heat shock proteins relevant to this thesis.

The heat shock response is one of the most highly conserved systems, it is rapid, intense and is found in all organisms examined from archaebacteria to humans (Lindquist and Craig, 1988). The expression patterns vary between different cell types and tissue types and between different conditions (Blake et al., 1990). However, it is now well established that HSP expression correlates with resistance to stress and that a species threshold for induction of the HSPs depends upon the conditions it is naturally exposed to (Lindquist and Craig, 1988; Feder and Hofmann, 1999). Thermotolerance closely parallels HSP accumulation, decay of thermotolerance parallels degradation of the HSPs and thermotolerance can be induced by exposure to other types of stress that induce HSPs. The degree of protection corresponds not with the type of stress but with the amount of HSP induction and thermotolerance and thermosensitivity during development correlate with the expression of HSPs. Overexpression of the HSPs

produces stress tolerance and when HSP expression is blocked thermotolerance is blocked (McAlister and Finkelstein, 1980; Tobe et al., 1984; Lindquist, 1986; Carper et al., 1987; Amin et al., 1995). It is worth noting that individual HSPs don't always protect against all types of stress presumably because different HSPs have different and separate functions.

Heat shock proteins have both house keeping functions and key roles in the cellular response to stress. Heat shock proteins have been shown to have functions in many cellular processes such as: cell proliferation and differentiation, protein trafficking, proteolysis, folding and assembly of proteins, disassembly of protein aggregates and degradation of abnormal or damaged proteins (Becker and Craig, 1994; Kusmierczyk and Martin, 2001). In the house keeping capacity of mediating protein folding, refolding, assembly, disassembly, degradation and protein trafficking the heat shock proteins are acting as molecular chaperones, although, not all HSPs are molecular chaperones and not all molecular chaperones are heat shock proteins (Ellis and Hartl, 1999). The presence of denatured proteins is an induction signal for HSPs and overloading or inhibition of the ubiquitin system also induces HSPs (Finley et al., 1984; Ananthan et al., 1986). During protein synthesis and translocation polypeptides are unfolded and therefore reactive regions of the protein that would normally be hidden by secondary and quaternary structure are exposed and could undergo deleterious interactions with other proteins. To prevent this from happening heat shock proteins bind to the unfolded proteins to protect them and facilitate folding of the polypeptide into the correct conformation. Heat and other stresses can cause proteins to become damaged or unfolded; it is one of the functions of HSPs to protect such proteins from deleterious interactions during periods of stress and then to refold them or transport them to the protein degradation machinery in the cell when conditions have returned to normal.

Increased ubiquitin mediated degradation can restore normal heat shock resistance in heat sensitive yeast cells demonstrating that the removal of misfolded and aggregated proteins is the key function of heat shock proteins in heat stress resistance in yeast (Friant et al., 2003).

During the stress response, translation is inhibited to reduce the accumulation of damaged and unfolded proteins, this occurs through the phosphorylation of the α subunit of the translation initiation factor 2 (eIF2α) by one of the eIF2α kinases. The phosphorylation of eIF2α inhibits eIF2B preventing GDP-GTP exchange and therefore inhibits translation (Prostko et al., 1992). PERK (protein kinase regulated by RNA (PKR) like ER kinase) appears to be the main candidate. It is activated upon endoplasmic reticulum stress but PKR can also be activated in response to cellular stress (Harding et al., 1999). Inhibiting translation reduces the pressure on the endoplasmic reticulum to process the proteins, further increasing the cells resistance to stress (Brostrom and Brostrom, 1998; Kaufman, 1999; Mori, 2000; Patil and Walter, 2001). HSP 90 appears to be involved in this process as it binds to eIF2α (Rose et al., 1987). GADD34 (Growth arrest and DNA damage 34), a stress inducible regulatory subunit of a holophosphatase complex, dephosphorylates eIF2α late in the stress response to facilitate translational recovery and the *de novo* expression of stress induced genes (Novoa et al., 2003).

Genotoxic stress induces the release of cytochrome c from mitochondria and the subsequent formation of a cytosolic complex with Apaf-1 gives rise to activation of procaspase 9. Caspase 9 then cleaves procaspase 3 to activate it and trigger apoptosis. The heat shock proteins are also involved in inhibiting apoptosis. HSP 70 can also bind to the mitochondrial intermembrane protein apoptosis inducing factor (AIF) to prevent apoptosis. AIF is a caspase independent death effector, so HSP 70 can block different apoptotic pathways making it a very potent anti-apoptotic factor (Ravagnan et al., 2001). HSP 70 binds to Apaf-1 (Beere et al., 2000; Saleh et al., 2000) and therefore prevents caspase-9 maturation. HSP 70 inhibits the pro-apoptotic kinase JNK as well as preventing caspase-3 maturation (Mosser et al., 1997; Gabai et al., 1998; Li et al., 2000; Park et al., 2001). HSP 27 binds to cytochrome c to prevent the interaction of procaspase-9 with Apaf-1 and therefore, like HSP 70, inhibits cytochrome c mediated apoptosis (Bruey et al., 2000). DNA damage, if severe enough, causes the dissociation of HSP 27 to allow caspase 3 activation and cell death (Pandey et al., 2000).

The heat shock proteins have a role in stabilizing protein kinases, allowing them to remain active for longer before they are degraded. HSP 70 binds to unphosphorylated

protein kinase C to stabilize it, which allows it to be re-phosphorylated. HSP 70 also binds to protein kinase B (Akt) and protein kinase A. HSP 90 binds to a number of protein kinases including Src, raf and ERK, and like HSP 70 it protects protein kinase B from dephosphorylation (Sato et al., 2000; Gao and Newton, 2002).

1.2.1 Regulation of the heat shock response

In eukaryotes, transcriptional, translational and post translational mechanisms are all employed in the regulation of the heat shock proteins. In E.coli there is a separate sigma factor, σ^{32}, that directs RNA polymerase to the heat shock promoters (Landick et al., 1984; Grossman et al., 1984). In humans there are three heat shock transcription factors (HSF) involved in regulating stress induced transcription, HSF-1, HSF-2 and HSF-4. In mice there are two HSF proteins, HSF-1 binds to DNA in stressful conditions and HSF-2 binds DNA constitutively but loses DNA binding activity during stressful conditions (Sarge et al., 1991). HSF-1 is essential for activation of HSPs in mammals (McMillan et al., 1998; Morimoto, 1998), when the cell is exposed to stressful conditions it activates the transcription of heat shock proteins by binding to the heat shock element (HSE) in the promoter regions of the heat shock genes. The consensus sequence for the HSE is a minimum of two inverted repeats of the pentamer 5'-nGAAn-3', HSF-1 and HSF-2 bind to the inverted repeats through major groove interactions (Kroeger et al., 1993). There is variation in the size of HSF and there is not a high level of homology, although all HSFs have two highly conserved features, an amino terminal DNA binding domain and a motif of heptad repeats that mediates oligomerization and transactivation (Sorger and Nelson, 1989; Clos et al., 1990; Jakobsen and Pelham, 1991; Wu, 1995). A loop within the DNA binding domain of HSF-1 dictates isoform specific DNA binding possibly through protein-protein interactions. The loop suppresses trimer formation under basal conditions and is required for heat inducible trimerization in vitro (Ahn et al., 2001). Interestingly different HSF-1 domains appear to be required for transient and sustained transcription (Sorger, 1990). HSF-2 is activated during specific stages of development (Sistonen et al., 1992). There are two isoforms of both HSF-1 and HSF-2, HSF-1 α and β and HSF-2 α and β (Goodson et al., 1995; Fiorenza et al., 1995) and HSF-2α has a role in enhancing stress induced HSF-1 mediated transcription (He et al., 2003). HSF-4

is only expressed in some tissues and represses HSF-1 mediated transcription (Zhang et al., 2001). HSF-3 has only been found in birds.

HSF-1 is already present in the cell allowing rapid activation and it produces a rapid increase in HSP transcription (Wu et al., 1987; Zimarino and Wu, 1987). Activation of HSF-1 leads to the formation of homotrimeric complexes, the monomers associate through the trimerization domain. Trimerization orientates the individual DNA binding domains so that they bind the inverted repeats of the HSE co-operatively (Sorger and Nelson, 1989; Peteranderl and Nelson, 1992; Ahn et al., 2001). Human HSF-1 is consitutively phosphorylated but further phosphorylation, of Ser230, induces transcriptional activity (Holmberg et al., 2001). Whilst heat shock results in the phosphorylation of HSF-1, the amino acid analogue azetidine induces oligomerization, acquisition of DNA binding activity and translocation to the nucleus but does not induce phosphorylation suggesting that phosphorylation is not required for the activation of HSF-1 but may be required for the maximum stimulation of transcription (Larson et al., 1988; Sorger and Pelham, 1988; Sarge and Morimoto, 1991; Sarge et al., 1991; Morimoto et al., 1992). HSF-1 has also been shown to repress the transcription of other proteins not involved in the stress response, especially proteins involved in the acute phase response (Cahill et al., 1996; Chen et al., 1997; Singh et al., 2000; Singh et al., 2002; Xie et al., 2002a; Xie et al., 2002b; Xie et al., 2003). Like the inhibition of translation of proteins not involved in the heat shock response this appears to reduce the burden of activity on the cell and therefore probably increases stress resistance.

As well as the rapid transcriptional activation by HSF-1 the HSP genes are preset in an open chromatin configuration (Wu, 1980; Keene et al., 1981). In some cases, for instance HSP 70, polymerase is already engaged on the genes and RNA synthesis is paused until the cell is exposed to stressful conditions to make the cells respond as rapidly as possible (Gilmour and Lis, 1986; Rougvie and Lis, 1988). HSP mRNAs are translated with high efficiency and the translation of other mRNAs is repressed by HSP 27, see 1.2.4 (Storti et al., 1980; Lindquist, 1981; Cuesta et al., 2000). HSP mRNAs are also stabilized by heat shock (Theodorakis and Morimoto, 1987; Petersen and Lindquist, 1988).

Under basal conditions HSF-1 monomers have low DNA binding activity and they are sequestered by intra- and intermolecular interactions to prevent trimerization in physiological conditions (Zimarino and Wu, 1987; Rabindran et al., 1993; Zuo et al., 1994; Wu, 1995; Orosz et al., 1996; Farkas et al., 1998; Liu and Thiele, 1999; Manalo et al., 2002). The heat shock proteins are themselves involved in the regulation of HSF-1 activity by providing a negative feedback mechanism. Heat stress activates HSF-1, by inducing trimerization and phosphorylation, HSF-1 then binds to the HSEs in the HSP promoters. In the proposed model the level of HSP 70 rises and once there are sufficient levels the amount of free HSP 70 increases. The free HSP 70 binds to HSF-1 to inhibit it and the heat shock response will start to subside. As damaged proteins accumulate free HSP 70 is used up, HSF-1 is released by HSP 70 removing the negative regulation of HSF-1, HSP 40 and HSP 90 are also involved. HSP 90 appears to be involved in trimer disassembly (DiDomenico et al., 1982; Craig and Gross, 1991; Baler et al., 1992; Abravaya et al., 1992; Zou et al., 1998; Duina et al., 1998; Ali et al., 1998; Shi et al., 1998; Bharadwaj et al., 1999; Marchler and Wu, 2001). HSF-1 activity is also negatively regulated by heat shock binding protein 1 (HSBP1) (Satyal et al., 1998), HSBP1 only binds to trimeric HSF-1. Increasing HSBP1 expression led to decreased survival after stress and decreasing HSBP1 increased survival after stress (Morimoto, 1998). The co-chaperone CHIP (C-terminus of HSP 70 interacting protein) is involved in the positive regulation of HSF-1 activity (Dai et al., 2003). The signal transduction and activators of transcription (STAT) proteins are also involved in the regulation of HSF-1, STAT-1 enhances HSF-1 mediated activation of transcription whereas STAT-3 antagonizes HSF-1 (Stephanou et al., 1998b; Stephanou et al., 1999).

In HSP 27, HSP 90 and HSP 40 there is an HSE in the first intron, which has been proposed to be part of a negative feedback mechanism. The presence of the intronic HSE in an artificial expression system reduces the expression of the reporter gene significantly and mutation of the intronic HSE partially restores the expression levels (Cooper et al., 2000). The intronic HSE binds HSF-1 *in vitro*, the HSP 90 intronic HSE binds HSF-1 with high affinity, however, in the HSP 40 gene the intronic HSE does not appear to be functional. It is not known whether the intronic HSEs are functional *in vivo*

but the conservation of the HSP 27 intronic HSE in mouse, rat and human as well as its presence in other heat shock proteins suggests that it could be (Shen et al., 1997).

There is evidence that HSF-1 is involved in the constitutive expression of HSPs as well and that it is required under normal physiological conditions to maintain redox homeostasis. HSF-1 is clearly an important regulatory protein so it is not surprising that HSP expression and the cellular homeostatic balance were affected by its absence in the HSF-1 knockout mice (Yan et al., 2002). The absence of HSF-1 could also affect the levels and activity of the other members of the HSF family.

1.2.2 Co-chaperones

The activity of the heat shock proteins is influenced by co-chaperones. A chaperone binding motif found in several co-chaperones as a degenerate 34 amino acid repeat called a tetratricopeptide repeat or TPR. Competition between co-chaperones to bind to HSP 70 and HSP 90 may modulate quality control for the cell determining when proteins should be refolded and when they should be destroyed (Schneider et al., 1996; Demand et al., 1998; Hohfeld et al., 2001). The interactions of HSP 70 and HSP 90 with some of the co-chaperones are shown in figure 1.1. There is competition between the co-chaperones for access to overlapping binding sites.

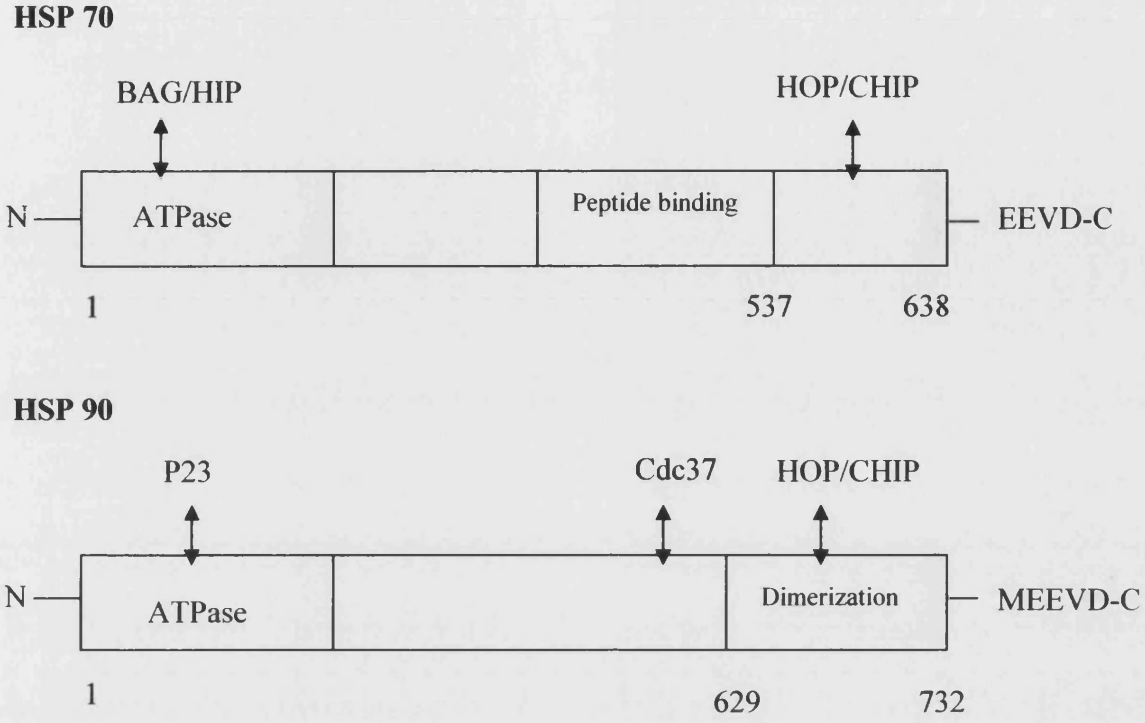


Figure 1.1 The structure of HSP 70 and HSP 90 and their interactions with different co-chaperones. Adapted from Nollen and Morimoto (2002).

The co-chaperone HOP (HSC 70-HSP 90 organizing protein) contains multiple TPR domains allowing it to bind to HSP 70 and HSP 90 simultaneously and assists chaperone mediated protein folding. It facilitates functional cooperation between HSP 90 and HSP 70. HSP 90 binds to HOP with high affinity and this is not affected by HSP 70 although the binding affinity of HSP 70 for HOP is increased by the presence of HSP 90. HOP inhibits ATP and p23 binding to HSP 90 unless HSP 70 is present in the complex. HOP therefore appears to act as a mediator of conformational change for the HSP 70 and HSP 90 proteins (Frydman and Hohfeld, 1997; Caplan, 1999; Hernandez et al., 2002).

CHIP is another co-chaperone that can bind to either HSP 70 or HSP 90. *In vitro* data suggests that CHIP inhibits the protein folding activity of the chaperones, although there is data suggesting that *in vivo* it increases the protein folding ability of HSP 70

(Ballinger et al., 1999; Connell et al., 2001; Kampinga et al., 2003). The C-terminus of CHIP is structurally similar to components of the ubiquitin/proteasome system. CHIP also posses a U-box domain that has been shown to participate in ubiquitin conjugation and a TPR tandem repeat. This may therefore link the molecular chaperones to the cells degradation machinery. Association of CHIP with HSP heterocomplexes bound to the glucocorticoid receptor leads to the dissociation of p23, ubiquitin conjugation and the degradation of the hormone receptor (Connell et al., 2001; Hohfeld et al., 2001), p23 appears to stabilize HSP 90 – glucocorticoid receptor interactions (Morishima et al., 2003). CHIP knockout mice are temperature sensitive and suffer high levels of apoptosis in multiple organs after stress (Dai et al., 2003).

The HSP 70 co-chaperone BAG-1 links HSP 70 to the ubiquitin/proteasome system (Luders et al., 2000a). BAG-1 binds to the amino terminal ATPase domain of HSP 70 to inhibit the chaperone activity and therefore competes with HIP whereas CHIP binds to the C-terminus (Takayama et al., 1997; Ballinger et al., 1999; Sondermann et al., 2001). There is some evidence that BAG-1 can negatively or positively regulate HSP 70 activity depending on the concentration of the BAG-1 protein. BAG-1 stimulates ADP dissociation of both HSP 70 and HSC 70 and therefore decreases affinity for the substrate, however, at low concentrations BAG-1 accelerates ATP triggered substrate release. Since different isoforms of the BAG-1 protein are expressed it seems likely that the concentration dependent effect actually reflects different levels of the different isoforms. BAG-1M inhibits protein refolding and BAG-1S stimulates protein refolding (Hohfeld and Jentsch, 1997; Luders et al., 2000b; Gassler et al., 2001). There are also other members of the BAG family, for instance BAG-3 which inhibits HSP mediated proteasomal mediated degradation (Doong et al., 2003).

In direct competition with BAG-1 is the co-chaperone HIP (HSC 70 interacting protein), HIP binds to the same site in the ATPase domain of HSP 70 but stabilizes the substrate bound state to increase the chaperone activity (Hohfeld et al., 1995; Nollen et al., 2001). HIP is a 41.3 KDa cytoplamic protein involved in regulating HSC 70 and in the assembly of the progesterone receptor (Hohfeld et al., 1995; Prapapanich et al., 1996). It has a 25KDa N-terminal domain that mediates dimerization and an 18KDa globular C-terminal domain. Both domains are involved in binding to HSC 70 (Velten et al., 2002).

HSBP1, as well as being invovlved in the regulation of HSF-1, alters the conformation of the HSP 70 ATPase domain and therefore inhibits HSP 70 mediated protein folding (McLellan et al., 2003). There is also another HSP 70 co-chaperone called RAP46 (Receptor associating protein of 46KDa), since RAP46 binds to both HSP 70 and HSC 70 and the HSP 70 family is the only family of proteins shown to bind directly to RAP46 the alternative name HAP (HSP70/HSC70 associating protein) has been proposed (Zeiner et al., 1997).

Cdc 37 is an HSP 90 co-chaperone, it is phosphorylated and the phosphorylation is required for it to bind to kinases and stimulate HSP 90 activity (Shao et al., 2003). Another HSP 90 co-chaperone is HARC (Hsp 90 associating relative of cdc37), it is a 35KDa protein expressed in the cytoplasm of cells in human tissue including the liver, skeletal muscle and kidney. HARC is also phosphorylated and it can form complexes with HSP 90, HSP 70, HOP and immunophilins but not p23. The function of this co-chaperone has not yet been established (Scholz et al., 2001). There are other HSP 90 co-chaperones including XAP2 (X associated protein) that modulates the subcellular localization of the transcription factor aryl hydrocarbon receptor (Ahr), and Aha1 (activator of HSP ATPase). Aha1 activates HSP 90 by targeting the rate limiting association of the N-terminal domains (Panaretou et al., 2002; Petrulis et al., 2003).

1.2.3 HSP 70 and HSP 90

HSP 70

At least ten HSP 70 related proteins have been found in the human genome but it is not known how many of these are functional (Mues et al., 1986). HSP 70 genes have at least 50% amino acid identity, however, some HSP 70 family genes are more similar to genes from other species than genes in the HSP 70 family from the same species. This suggests that during evolution there were early gene duplication events followed by conservation of the HSP 70 family (Lindquist and Craig, 1988; Boorstein et al., 1994). The E.coli HSP 70 gene DnaK, so called because it stops DNA synthesis at high temperatures, is required for growth at high temperatures. The three most well characterized members of the family are: HSP 70 (also called HSP 73), HSC 70 (also

called HSP 72) and glucose regulated protein 78 (grp78, also called BiP) (Shiu et al., 1977; Munro and Pelham, 1986; Lindquist and Craig, 1988). HSP 70 is expressed basally at low levels and in stressful conditions at high levels, HSC 70 is expressed at high levels basally and is slightly heat inducible and grp78 also has a high level of basal expression (Lee et al., 1984; Brown et al., 1993). Another member of the family is found in the mitochondria (mtHSP70), in fact there are many molecular chaperones expressed in the mitochondria including mtHSP 70, HSP 60, HSP 10, mtgrpE and mtDNAJ which together produce a mitochondrial specific stress response (Ryan et al., 1997; Zhao et al., 2002). During stress large amounts of HSP 70 move to the nucleus. In *Drosophila* it is also found at the cell membranes, and during recovery it returns to the cytoplasm (Welch and Suhan, 1985; Collier and Schlesinger, 1986). HSC 70 is cytoplasmic but mainly concentrated around the nucleus. About 5% of dnaK is phosphorylated and the protein is capable of self phosphorylation (Zylicz et al., 1983; Zylicz and Georgopoulos, 1984).

HSP 70 interacts with HSP 40 and the exsistence of multiple isoforms of each of the proteins could mean that there are many HSP 70-HSP 40 combinations each with its own substrate specificity or chaperone activity (Lu and Cyr, 1998).

HSP 70 has many roles as a molecular chaperone. It is involved in the folding and refolding of proteins, it stabilizes unfolded precursor proteins prior to assembly and during translocation into the ER and the mitochondria and it is also involved in the rearrangment of protein oligomers. Grp78 is involved in the translocation of proteins into the ER, as well as binding to unfolded or misfolded proteins. HSC 70 also binds to nascent polypeptides on ribosomes and has prolonged interactions with proteins that do not fold correctly (Rothman, 1989; Beckmann et al., 1990; Vogel et al., 1990; Becker and Craig, 1994). HSP 70 proteins all bind to ATP and have ATPase activity (Zylicz et al., 1983; Welch and Feramisco, 1985; Clarke et al., 1988; Liberek et al., 1991a). It has been shown to be the N-terminal of the protein that binds to ATP and has ATPase activity whereas it is the C-terminal of the protein that binds to the polypeptide. The binding and release of peptides is dependent on the binding and hydrolysis of ATP and is accompanied by a conformational change in HSP 70 (Flynn et al., 1989; Liberek et al., 1991b). A role has been proposed for HSC 70 in tubulin folding and the assembly

and disassembly of the mitotic apparatus (Agueli et al., 2001). It is also involved in uncoating clathrin coated vesicles although it undoubtedly has other functions as well such as its involvement in ubiquitin mediated degradation (Schlossman et al., 1984; Chappell et al., 1986; Chappell et al., 1987; DeLuca-Flaherty et al., 1990; Bercovich et al., 1997).

HSP 70 is involved in the folding of proteins and there is evidence that HSP 60 and HSP 70 act sequentially in a folding and assembly pathway where HSP 70 recognizes the polypeptide backbone of the unfolded protein and facilitates the formation of a folding intermediate which is then recognized by HSP 60. (Cheng et al., 1989; Kang et al., 1990; Manning-Krieg et al., 1991; Langer et al., 1992; Gragerov et al., 1992).

Another role has been proposed for HSP 70 in presenting signal sequences to the correct receptors as a signal recognition particle (SRP) independent pathway for targeting precursor proteins not cotranslationally targeted by SRP to the correct destination. HSP 70 has been compared structurally to the human leucocyte antigen (HLA) class I antigen presenting molecule at the primary sequence and secondary structure levels (Hann and Walter, 1991; Rippmann et al., 1991; Dingwall and Laskey, 1992). HSC 70 is involved in targeting class II MHC molecules to endocytic compartments (Lagaudriere-Gesbert et al., 2002).

HSP 90

The HSP 90 protein is well conserved, all eukaryotic HSP 90 proteins have a least 50% amino acid identity and 40% amino acid identity with the *E. coli* protein (Farrelly and Finkelstein, 1984; Bardwell and Craig, 1987).

HSP 90 is abundant at normal temperatures and induced by stress as well. Like HSP 70 there is a constitutive and an inducible form of the protein. HSC 90 is expressed at high levels in normal conditions and is moderately heat inducible whereas HSP 90 is expressed at lower levels in normal conditions and is highly heat inducible. In *S.cerevisiae* both HSP 83 and HSC 83 (HSP 90 and HSC 90) are required for normal growth at high temperatures and cells with both HSP 83 and HSP 83 mutated do not grow at any temperature (Borkovich et al., 1989). HSP 90 is predominantly cytoplasmic

and partially relocalizes to the nuclei during heat shock (Lai et al., 1984; Collier and Schlesinger, 1986). In vertebrate cells HSP 90 has a signal sequence to transport it across the endoplasmic reticulum (ER). The protein found in the ER (glucose regulated protein 94 or grp94) is larger than the cytosolic form with an apparent molecular weight on an SDS-PAGE gel of around 94KDa, whereas the cytoplasmic protein has an apparent molecular weight of 87-92KDa. Another member of the family ERp99 is approximately 92KDa and is a membrane spanning protein in the ER (Shiu et al., 1977; Mazzarella and Green, 1987). HSP 90 possesses low endogenous peptidase activity and undergoes long term autocatalytic digestion, it has also been be detected as a 230KDa multimeric form and a 73KDa truncated form (Montel et al., 2000). HSP 90 has an ATP/ADP binding site with affinities that would allow ATP/ADP cycling in normal cellular conditions. ATPase activity has been shown and it seems likely that ATP binding and hydrolysis cause a conformational change in the HSP 90 protein dimer allowing cycles of substrate binding (Nadeau et al., 1993; Prodromou et al., 1997).

Cytosolic HSP 90 has been shown to bind to retroviral transforming proteins, cellular tyrosine kinases, steroid hormone receptors, actin and tubulin (Lindquist and Craig, 1988; Pratt, 1997). HSP 90 can be phosphorylated in all organisms investigated, which appears to be a consequence of its association with tyrosine kinases.

HSP 90 associates with actin (Nishida et al., 1986; Koyasu et al., 1986). Since HSP 90 moves into the nucleus during heat shock and actin filaments rearrange during heat shock and are found in the nuclei of heat shocked cells, it seems likely that the association of HSP 90 with actin provides a transport mechanism (Welch and Suhan, 1985).

HSP 90 binds to raf-1 and is involved in activating it but the interaction requires the co-chaperone cdc37 (Grammatikakis et al., 1999). Prolonged exposure to the HSP 90 inhibitor geldanamycin causes dissociation of raf-1/HSP 90 complexes resulting in enhanced degradation of raf-1 and therefore reduced raf-1 activity. However, short exposure to geldanamycin leads to raf-1 activation. One possible explanation for this is that HSP 90 is required for the maturation and maintenance of raf-1, but raf-1 needs to be released for it to be activated by other regulators (Nollen and Morimoto, 2002).

Co-operative functions of HSP 70 and HSP 90

Both HSP 90 and HSC 70 bind to and stabilize both mutant and wildtype p53 (Pinhasi-Kimhi et al., 1986; Zylicz et al., 2001; King et al., 2001). A model has been proposed where both mutant and wildtype p53 can be complexed with HSC 70 and HSP 40 in normal conditions, supressing the oligomerization of p53. However, in the presence of HSP 90 and HOP the wildtype p53 binds to HSP 90, this complex can be dissociated by BAG-1 to allow free p53 that can then be targeted for proteolysis. During stressful conditions HSP 70 levels increase allowing HSP 70/BAG-1 complexes to form, thus BAG-1 could be sequestered allowing the import of HSP 90/p53 complexes into the nucleus. Potentially chaperones could bind mutant p53 to stabilize and sequester it, thereby inactivating it by preventing proteolysis and import into the nucleus. Both HSP 70 and the mitochondrial HSP 70 protein grp 75, also called mtHSP70 can bind to p53 (Wadhwa et al., 2002).

HSP 70 and 90 also have cooperative roles in delivering preproteins to the mitochondria. They bind to the mitochondrial membrane receptor Tom70 via their TPR domains (Young et al., 2003).

HSP 90 forms a heterocomplex with HSP 56 and HSP 70, the heterocomplex binds to steroid hormone receptors including estrogen, progesterone and glucocorticoid receptors in the cytoplasm to prevent them being activated in the absence of the hormone (Dougherty et al., 1984; Sanchez et al., 1986; Sanchez et al., 1987; Redeuilh et al., 1987; Mendel and Orti, 1988; Sanchez, 1990; Pratt, 1997).

1.2.4 HSP 27

Unlike HSP 70 the small HSPs have greater homology within species than between species. The small heat shock proteins all share a homologous C-terminal region called the α-crystallin domain. It is 80-100 residues long and has chaperone like activity *in vitro* (Lindquist and Craig 1988). The small heat shock proteins have structural plasticity in solution and this plasticity may be important for recognizing a diverse array of target proteins (Haley et al. 2000).

HSP 27 has both phosphorylation dependent and phosphorylation independent functions. The activity of HSP 27 as a molecular chaperone is not dependent on phosphorylation whereas its ability to stabilize actin is phosphorylation dependent. In normal conditions HSP 27 is predominantly found in the cytoplasm, it can be detected in the nucleus after stress and also forms polymeric structures outside the nucleus sometimes called heat shock granules (Kim et al., 1984; Rollet and Best-Belpomme, 1986; Collier and Schlesinger, 1986; Huot et al., 1995). In *Drosophila* there are different isoforms of HSP 27 that are phosphorylated and expressed in different developmental stages and in different conditions (Marin et al., 1996)

During heat shock, HSP 27 binds to the translational adapter protein eIF4G and therefore inhibits the formation of the cap binding protein initiation complex eIF4F and translation. eIF4F is required for the translation of most mRNAs, the cap binding complex unwinds 5' secondary structure. The inhibition of protein synthesis during heat shock prevents the accumulation of proteins that cannot be processed by the cell, proteins that have not been folded yet have reactive regions exposed that may undergo inappropriate interactions with other proteins. Normally Cap dependent translation is activated by the phosphorylation of the cap binding complex component eIF4E by the kinase Mnk1. Mnk1 is not inhibited during heat shock but does not need to be because it can only phosphorylate eIF4E when eIF4E is bound to eIF4G. When HSP 27 binds to eIF4G it traps it in insoluble heat shock granules around the nucleus. Under normal conditions both HSP 27 and eIF4G are cytoplasmic. Heat shock can result in a tenfold reduction in the abundance of soluble eIF4G (Cuesta et al. 2000). The heat shock proteins are still translated, this could be because the polymerase is already bound to the genes, see 1.2.1. The endoplasmic reticulum HSP 70 protein grp78 (BiP) also has an internal ribosome entry site that allows cap-independent translation initiation therefore bypassing the need for eIF4G to translate this particular HSP (Macejak and Sarnow, 1991).

1.2.5 HSP 56

HSP 56 (also called FKBP52, FKBP56, FKBP59, HBI, p59 and p56) is a peptidyl prolyl cis-trans isomerase (PPPIase) (Chambraud et al., 1993). HSP 56 has an important catalytic role in protein folding because the isomerization of proline is a slow step in protein folding. In intact cells HSP 56 is mostly localized in the nucleus with some colocalization to the microtubules in the cytoplasm. It contains a negatively charged domain and is thought to contain a nuclear localization recognition signal (Czar et al., 1995).

HSP 56 is an immunophilin, it binds to the glucocorticoids and they are immunosuppressants. HSP 56 also binds to a chemical immunosuppresant FK506 and has been implicated in mediating the immunosuppressant effect of FK506, possibly through the glucocorticoids (Yem et al., 1992; Ning and Sanchez, 1993). However whether the binding of HSP 56 to FK506 is responsible for the immunosuppressive effects of FK506 has not been shown.

HSP 56 forms a heterocomplex with HSP 90 and HSP 70 to regulate steroid hormone receptor activation (Sanchez et al., 1990). It appears that HSP 56 binds reversibly to HSP 90 but does not bind to HSP 70, suggesting that HSP 56 and HSP 70 bind to different sites on the HSP 90 protein to form the complex rather than binding to each other (Czar et al., 1994), also see 1.2.3 co-operative functions of the HSPs. *In vivo* when HSP 56 is bound to HSP 90 it, as a prolyl isomerase, enhances the activity of glucocorticoids by increasing the hormone binding affinity of the glucocorticoid receptor (Riggs et al., 2003).

1.2.6 The protective effect of heat shock proteins.

Heat shock proteins enable cells to survive otherwise lethal conditions such as heat stress and hypoxia through a variety of mechanisms already discussed. The protective effect of heat shock proteins has been shown in many different systems against many different stresses and it is not possible to cover the full range of the literature here. Therefore a brief introduction to the protective effect of HSP 70, HSP 90 and HSP 27 in neuronal cells will follow.

Heat shock proteins protect embryonic, neonatal and young adult neurons in a variety of conditions. HSP 70 can protect embryonic and neonatal neurons against heat stress induced cell death. Importantly it can also protect synaptic function in neonatal brain slice cultures against stress induced damage (Mailhos et al., 1994; Wyatt et al., 1996; Kelty et al., 2002). HSP 90 protects neuronal cell lines and embryonic trigeminal neurons against thermal stress (Mailhos et al., 1994; Wyatt et al., 1996). HSP 27 expression has been shown to protect against both apoptotic and necrotic death in non-neuronal cells (Mehlen et al., 1995; Samali and Cotter, 1996). It also has a protective effect in neurons. In young adult dorsal root ganglia cells, sciatic axotomy induces the expression of HSP 27 in some cells and the expression of HSP 27 correlates with survival. HSP 27 expression is not induced in the neonatal sensory neurons after axotomy and only 5-10% of the cells axotomized survive. Overexpression of HSP 27 improves the survival of both sensory and sympathetic neurons after NGF withdrawal (Lewis et al., 1999). HSP 27 overexpression in transgenic mice reduces seizure severity, mortality, and hippocampal cell death induced by kainic acid, which is a model of epilepsy (Akbar et al., 2003). HSP 27 also protects against kainate induced hippocampal cell death when it is overexpressed by delivery in a herpes viral vector (Kalwy et al., 2003). HSP 56 has not been shown to have a protective effect against stress in neurons.

1. 3 Ageing

Ageing in living systems can be defined simply as the changes that occur in association with the flow of time. Senescence is the counterpart of ageing and can be broadly defined as the damage or loss of function associated with ageing. Senescence occurs both in cells that are still growing and dividing, known as replicative senescence, and in post mitotic cells. Senescent cells accumulate with age in tissues *in vivo* and therefore contribute to age related dysfunction (Dimri et al., 1995).

1.3.1 Replicative senescence

Replicative senescence appears to be an evolutionarily primitive process as it occurs in the replicating cells of many organisms ranging from humans to yeast (Mortimer and

Johnston, 1959), see Jazwinski (1990) for review. The theory of the Hayflick limit states that replicating cells become senescent after a set number of cell divisions, in human fibroblasts this is about fifty cell divisions (Hayflick and Moorhead, 1961; Hayflick, 1965). This, however, assumes that replication occurs in all cells at a more or less constant rate. Questions have been raised about the accuracy of this theory not least because there is a large variation in the replicative potential of sister cells (Smith and Whitney, 1980). One possible explanation for this is that fibroblasts are immortal but become committed to terminal differentiation, senescence and death over time. Senescent cells start to accumulate but a small population of fibroblasts replenish the population *in vivo* until all the immortal cells are eventually lost (Holliday et al., 1977).

Replicative senescence has frequently been used as a model to investigate ageing at the cellular level. It is a useful model of senescence since it can be more easily manipulated than senescence in postmitotic cells and the results are much easier to interpret because the whole ageing process is much more complex than replicative senescence. Replicative senescence has been proposed as a useful model for whole organism ageing because: cells from short lived species senesce after fewer doublings than comparable cells from longer lived species, cells from people with premature ageing syndromes senesce more rapidly than age matched controls and the regulation of at least some genes is altered similarly in replicative senescence in culture and ageing *in vivo* (Campisi et al., 1996). The frequently cited finding that cells from older donors senesce after fewer doublings than cells from younger donors, (Martin et al., 1970), depended on the use of refrigerated cadavers and demonstrated a weak relationship (Rubin, 2002). A large study using cells from living donors found no correlation between the replicative life span of fibroblasts and the age of the donor (Cristofalo et al., 1998). This conflict would be explained by the presence of a small population of immortal cells to replenish the fibroblast population as proposed by Holliday et al (1977). This does not eliminate the usefulness of replicative senescence as a model, there are still many similarities between ageing and replicative senescence, for instance the correlation with species lifespan and with premature ageing syndromes.

1.3.2 Theories of ageing

Many theories have been proposed to explain how and why organisms age. Most of these theories describe one of two mechanisms. One proposes that senescence is caused by some kind of cellular clock, telomere shortening being the most likely candidate, and the other proposes that the accumulation of damage to the cell triggers senescence. These two mechanisms will be reviewed briefly.

Telomere theory of ageing

Telomeres are repetitive sequences at the ends of chromosomes, they are necessary to prevent the ends of chromosomes fusing or becoming damaged. The telomeres are shortened with each round of DNA replication because a short length of DNA before the sequence to be copied is required for the binding of the RNA primer, this short sequence can not itself be copied by the normal DNA replication machinery and is lost (Olovnikov, 1973; Harley et al., 1990). The shortening of the telomeres was proposed as a cellular clock that could count down the replications and initiate senescence when they became too short. Telomeres appear to be able to trigger senescence when they reach a critical length (Allsopp and Harley, 1995).

Examinations of ageing cell populations *in vitro* on a cell by cell basis have revealed that cells are continuously dropping out of the cell cycle and that they do so with increasing frequency until the whole population ceases doubling. Even in late passage cultures the cells that are still going through the cell cycle, cycle as rapidly as those in early passage populations (Karatza et al., 1984). Although not inconsistent with the theory that the ageing process is regulated in some way, or with the concept that if telomeres become too short they can trigger senescence, it is inconsistent with the notion of a cellular clock that determines when senescence occurs because inherent to this theory is the idea that senescence does not occur before a set period of time, i.e. the 50 divisions suggested by Hayflick (1961) and Hayflick and Moorhead (1965). There are some other inconsistancies with this theory as well, there is heterogeneity in the length of telomeres (Zijlmans et al., 1997; Baird et al., 2003), and there is no consistent telomere length at which senescence occurs (Zhu et al., 1999; Ouellette et al., 2000). See Campisi et al. (2001b) for a comprehensive review of the telomere theory of ageing.

However, telomeres can not be ruled out completely as short telomeres can induce senescence (Allsopp and Harley, 1995) .

Most telomeres shorten gradually but there are also occasional substantial changes in telomere length, notably some telomeres are almost completely lost at senescence, whilst others remain quite long (Baird et al., 2003). It has been suggested that these occasional large changes in the lengths of telomeres are caused by sub-lethal stresses such as oxidative damage. Cells exposed to severe but sub-lethal stresses can enter a state called stress-induced premature senescence (SIPS). Overexpression of the catalytic subunit of human telomerase (hTERT) results in longer telomeres but does not prevent SIPS although it does reduce cell death suggesting that it is not telomere shortening that induces SIPS (Gorbunova et al., 2002).

In fact, it appears to be the structure of the telomere rather than the length that determines senescence (Rubio et al., 2002; Karlseder et al., 2002). The 3' single strand G-rich overhang at the end of the telomere appears to be essential in preventing senescence, and cell division appears to trigger senescence by eroding it (Stewart et al., 2003). A two state model has been proposed where the telomere is a dynamic nucleoprotein complex that can switch between being capped and being uncapped (Blackburn, 2000). Capping protects the end of the telomere from being seen as broken DNA and appears to be provided by Ku. Ku is a protein that forms part of the DNA damage response machinery (Porter et al., 1996; Gravel et al., 1998; Hsu et al., 1999). Uncapping and cap prevented recombination are proposed to occur when individual telomeres are short enough to cause cell cycle arrest allowing homologous recombination of the shortened telomeres to repair them. It appears to be an alternative mechanism for repairing shortened telomeres in the absence of telomerase (McEachern and Blackburn, 1996). Telomerase is an enzyme that can add nucleotides to the ends of telomeres to extend them, although it is only expressed in some tissues and the expression patterns differ between species (Prowse and Greider, 1995).

Free radical theory of aging

Telomere erosion certainly can trigger senescence, but this may not explain everything. Senescence doesn't just occur in replicative cells; it occurs in post mitotic cells as well

(Rattan, 1995; Ulfhak et al., 2002). Accumulation of oxidatively modified molecules was first proposed as a causative factor in ageing and age related degenerative diseases by Harman (1956). Ageing cells and organisms accumulate increased levels of oxidant damaged DNA and proteins (Rattan, 1995).

The amount of oxygen a cell is exposed to affects its' lifespan, supporting the idea that oxidation is a key factor in the accumulation of damage in cells. Cells grown in high oxygen concentrations have shorter life spans and increased telomere shortening (von Zglinicki et al., 1995), concomittantly cells grown in low oxygen concentrations have an increased life span (Packer and Fuehr, 1977). This is also true for whole organisms, the lifespan of *C.elegans* is increased when the worms are kept at lower oxygen concentrations (Honda et al., 1993).

Cells produce reactive oxygen species as byproducts of some reactions, and because these are unstable they react with the molecules around them causing damage to various components of the cells. They are produced by the mitochondria, peroxisomes, lipoxygenases, NADPH oxidase and cytochrome p450, and are also produced exogenously by UV light, ionizing radiation, chemotherapeutics, inflammatory cytokines and environmental toxins, for review see (Finkel and Holbrook, 2000). For a comprehensive review of the free radical theory of ageing see (Beckman and Ames, 1998). Mitochondria generate a large amount of oxidants and accumulate significant oxidative damage with age, as they are the "powerhouses" of the cell the accumulated damage has a significant impact on the ability of the cell to function (Shigenaga et al., 1994).

Reactive oxygen species can be detoxified by the superoxide dismutase (SOD) enzymes. If SOD1 or SOD2 expression is inhibited, senescence is induced but this is dependent, at least in the case of SOD1, on p53 induction (Blander et al., 2003). In stationary phase yeast cultures SOD1 and SOD2 mutants lose viability rapidly and the double mutant dies within a couple of days (Longo et al., 1996), *C.elegans* mutants with reduced SOD activity also have shortened lifespans (Honda et al., 1993). Overexpression of SOD1 in the motorneurons of *Drosophila* increases the life span of the flies by up to 40% (Parkes et al., 1998). p21 is a cyclin-dependent kinase inhibitor that mediates both p53 induced

growth arrest and p53 independent growth arrest. p21 has been shown to mediate reactive oxygen species induced senescence in fibroblasts and p21 expression is increased with age (Macip et al., 2002).

With increasing age there is reduced fidelity of protein synthesis (Luce and Bunn, 1989) and accumulation of oxidized proteins. Proteases are not activated, hence inactive or less active proteins and enzymes accumulate (Starke-Reed and Oliver, 1989). Changes in post-translational modification of proteins such as deamination, racemization, oxidation and glycation accumulate with age, for comprehensive reviews of the literature regarding age related changes in protein activity, conformation and modification see Levine and Stadtman (1996), Rattan (1996a) and Baynes (2000). Deamination and racemization of amino acids accumulate at similar rates in both long and short lived organisms suggesting that these are markers of ageing but are not determinants of ageing. In contrast glycation and lipoxidation appear not only to be biomarkers of ageing but to contribute to ageing and age related dysfunction as well. Glycation and lipoxidation are particularly toxic because they appear to act as amplifiers of oxidative damage. Reactive oxygen species damage molecules by reacting with them producing inert products. Moreover, when reactive oxygen species react with EAGLEs (Either advanced glycation or lipoxidation end products) the reactions produce more reactive oxygen species and therefore cause further damage (Baynes, 2000).

There are three types of DNA damage that occur in nuclear and mitochondrial DNA, they are: base mispairing; spontaneous chemical changes such as depurination and depyrimidation and induced chemical changes such as single and double strand breaks and crosslinks. A number of studies show accumulation of DNA damage during ageing, both mitochondrial and nuclear DNA are damaged but the accumulation of mitochondrial DNA damage is greater (Agarwal and Sohal, 1994; Ozawa, 1995), for review see (Rattan, 1995). There is evidence that the ability to repair DNA damage correlates positively with a species lifespan (Grube and Burkle, 1992). However, there does not appear to be an age related reduction in DNA repair capacity substantial enough to be a causative factor in ageing at a gross level (Rattan, 1989; Niedermuller, 1995). There is, however, preferential repair of active regions of the genome and the transcribed strand (Link, Jr. et al., 1992; Bohr and Anson, 1995), and work on fine

structure DNA repair has shown that there is intragenomic heterogeneity in DNA repair and mutation hot spots may be due to local repair deficiencies (Tornaletti and Pfeifer, 1994). At the level of individual genes there is some evidence for an age related decline in repair capacity (Wei et al., 1993).

There is good evidence that both telomere erosion and the accumulation of damage can trigger a senescent state in cells. A multifaceted mechanism of ageing seems increasingly more likely than a single mechanism. However, the discovery of single genes able to affect the lifespan of organisms suggests that there is a co-ordinated system to regulate ageing (Lithgow, 1996). The insulin/IGF-1 pathway appears to have evolved early with striking similarities between yeast, flies and worms. The pathway allows organisms to postpone reproduction in unfavourable conditions, therefore avoiding the energetically costly process of producing progeny when there are insufficient food resources to support it. It also promotes fat/glycogen storage as food sources decline and stimulates the stress response. Interestingly, the ability to detoxify reactive oxygen species is the only trait that could not be uncoupled from longevity in *C.elegans* (Lithgow et al., 1995; Kimura et al., 1997; Gems and Partridge, 2001; Kenyon, 2001). A thermotolerant, long lived srain of *C.elegans* expresses elevated levels of HSP 16, HSP 16 is homologous with mammalian HSP 27 (Walker et al., 2001). Lithgow et al (1995) have shown that when the resistance of *C.elegans* to stress is increased by environmental or genetic manipulation there is an increase in lifespan. They propose that the ability to respond to stress is the limiting factor in life expectancy. HSF-1 not only regulates heat shock protein expression but also appears to be involved in regulating ageing. HSF-1 overexpression increases life span and acts together with DAF-16 in the insulin/IGF-1 pathway to switch on the heat shock proteins as well as other crucial genes such as SOD (Hsu et al., 2003). Interestingly senescent fibroblasts do not express IGF-1 and fibroblasts immortalized by transfection with a temperature sensitive SV40 T antigen regained the ability to express IGF-1 (Ferber et al., 1993). Selecting for heat stress resistance in *Drosophila* led to a small but statistically significant increase in the longevity of male flies but curiously did not result in an increase in female longevity (Norry and Loeschcke, 2003).

1.3.3 Phenotypic changes that occur with age

There are many changes that occur in aged cells, some undoubtedly caused by reactive oxygen species damaging the cellular machinery. Although there is some variation there are general phenotypic changes that occur in most senescent cells. Senescent cells have ceased to divide, are larger and more irregular in shape and have larger nuclei and shorter telomeres. They exhibit increased lysosome biogenesis, decreased protein synthesis and protein degradation, they have altered gene expression and accumulate damaged proteins. Some cells express β-galactosidase once they have become senescent, this is termed senescence associated or SA-β-gal (Dimri et al., 1995; Campisi et al., 1996). Some of the key changes that occur in ageing cells are summarized in table 1.1.

Summary of the changes that occur with age	References
The ability of cells to grow and divide is lost Replicating cells cease dividing Telomeres shorten and become damaged	(Campisi et al., 1996) (Harley et al., 1990) (Campisi et al., 2001) (Stewart et al., 2003)
DNA becomes damaged Somatic mutations DNA methylation increases	(Rattan, 1995) (Wilson and Jones, 1983)
Proteins and protein expression are affected The fidelity of protein synthesis is reduced Transcription of genes involved in protein turnover is reduced Proteosomal activity is reduced Oxidized and ubiquitinated proteins accumulate Deamination, oxidation and glycation are all increased	(Chondrogianni et al., 2003) (Rattan, 1996) (Rattan and Clark, 1996) (Levine and Stadtman, 1996)
Signalling pathways are affected Caveolae accumulate and appear to affect signalling ERK-2 activity is reduced P53 activity is increased P21 activity is increased NF-kB exhibits increased DNA binding activity but decreased acute activation	(Cho et al., 2003) (Afshari et al., 1993) (Medrano et al., 1994) (Atadja et al., 1995) (Macip et al., 2002) (Ponnappan, 1998)
The stress response is impaired Basal levels of heat shock proteins are increased Acute activation of the heat shock response is impaired Decreased ability to maintain high levels of heat shock protein expression HSF-1 activity is reduced	(Lee et al., 1999) (Fonager et al., 2002) (Udelsman et al., 1993) (Blake et al., 1991b) (Hall et al., 2000) (Fawcett et al., 1994)

Table 1.1: Summary of some of the cellular and molecular changes that occur during ageing.

Changes in signalling pathways

Many changes in cellular signalling occur during ageing, some of them are undoubtedly involved in producing the phenotypic changes that occur during ageing and some, since the signalling pathways are connected, cause other changes to occur. Some of these changes in signalling pathways will now be discussed.

ERK, p38 MAPK, PI-3-kinase, NF-κB, p53 and the heat shock response have all been shown to be activated by oxidant injury and show diminished activity with age, for review see (Finkel and Holbrook, 2000). PI-3 Kinase is one of the downstream components of the insulin/IGF-1 pathway in *C.elegans* (Kenyon, 2001). ERK activity is reduced with age whereas JNK activity is not reduced with age (Liu et al., 1996; Guyton et al., 1998). One of the roles of HSP 70 is to block the stress induced apoptotic kinase JNK (Gabai et al., 1998). Since HSP 70 expression is reduced with age (see 1.3.4), the inhibition of this kinase is reduced.

The hydrogen peroxide induced activation of ERK and Akt is reduced in aged hepatocytes compared to young hepatocytes, and interestingly this age related loss of activity is partially alleviated by caloric restriction (Ikeyama et al., 2002). Replicatively senescent human melanocytes are unable to phosphorylate ERK-2 (p42 MAPK), as are senescent human fibroblasts, and correspondingly there is no nuclear accumulation of ERK-2 (Afshari et al., 1993; Medrano et al., 1994). However, ERK-1 (p44 MAPK) is phosphorylated in stimulated and unstimulated senescent fibroblasts. Both ERK-1 and ERK-2 are expressed at the same level in young and senescent fibroblasts (Afshari et al., 1993).

NF-κB on the other hand exhibits increased DNA binding activity with age in normal conditions in a variety of tissues (Supakar et al., 1995; Helenius et al., 1996a; Helenius et al., 1996b; Poynter and Daynes, 1998) but like the heat shock response acute activation of NF-κB is reduced with age (Trebilcock and Ponnappan, 1996), for review see (Ponnappan, 1998).

P53, as the guardian of the genome, has an important role in senescence, triggering apoptosis when the cells' DNA has been damaged beyond repair. The activity of p53

increases in senescent cells and appears to be a trigger for replicative senescence (Atadja et al., 1995; Bond et al., 1996; Webley et al., 2000); inactivation of p53 delays senescence and can return senescent cells to S phase (Bond et al., 1994; Gire and Wynford-Thomas, 1998). Expression of a consitutively active mutant form of p53 results in early onset of the ageing phenotype in mice (Tyner et al., 2002).

Changes in protein expression and degradation

Caveolin 1 and 2 are integral membrane proteins that are the main component of caveolae, which are vesicular organelles formed from the subdivision of the plasma membrane, for review see Severs (1988). They have been proposed to act as compartments to store signalling molecules and provide areas where receptors can be concentrated, and could therefore regulate activation and cross-talk between signalling cascades (Anderson, 1993; Lisanti et al., 1994). There appears to be reciprocal negative regulation between caveolin-1 and p42/44 MAPK (Engelman et al., 1998). Cho et al (2003) show that reducing the level of caveolin-1 in senescent cells restored normal growth factor stimulation of the cells, including restoring the normal phosphorylation and translocation of ERK. DNA synthesis is resumed and the cells re-enter the cell cycle, associated with corresponding reductions in the levels of p53 and p21.

As previously discussed, oxidatively damaged proteins accumulate with age and proteins are damaged continuously throughout the life of the cell, but in young cells most of the damaged proteins are removed by the normal processes of protein turnover. The proteasome is therefore an important component in the cells' defence. Senescent fibroblasts have reduced levels of proteasomal peptidase activities, increased levels of oxidized and ubiquitinated proteins, and the half lives of undamaged proteins are increased (Ishigami and Goto, 1988; Ishigami and Goto, 1990). Inhibiting the proteasome in young cells induces a senescent like phenotype and overexpressing the β1 or β5 catalytic subunits of the proteasome increased proteasomal activity and increased the cells capacity to cope with oxidative stress (Chondrogianni et al., 2003). Although there is some loss in the activity of the 20S proteasome it is not sufficient to account for the decreased protein degradation with age (Shibatani et al., 1996). Another important part of the protein degradation machinery is the lysosome. The chaperone HSC 73 plays

an important role in one of the lysosomal pathways of protein degradation; it binds to the substrate and directs it to the lysosomal membrane (Chiang et al., 1989; Terlecky and Dice, 1993) where it is bound by the glycoprotein receptor lgp96, also called lamp2a (lysosome associated membrane protein 2a). The substrate is then transported into the lysosome by lysosome associated HSC 73 (ly-HSC 73) (Cuervo and Dice, 1996). Approximately 30% of the cytosolic proteins are degraded through this pathway during stressful conditions and it also becomes impaired in old age (Cuervo and Dice, 1998). In this instance it is the loss of lamp2a expression which results in the age related impairment (Cuervo and Dice, 2000). Caloric restriction reduces the accumulation of damaged proteins in aged animals (Takahashi and Goto, 1987). There is also a decrease in free ubiquitin and an increase in ubiquitinated proteins with age, although there is no evidence that the ability of senescent cells to degrade ubiquitinated proteins per se is impaired with age (Pan et al., 1993). Interestingly ubiquitin overexpression overcomes the requirement for ceramide synthesis and heat shock protein expression for resistance to heat shock in yeast (Friant et al., 2003).

Changes in DNA methylation

A decrease in the amount of DNA methylation has been correlated with ageing (Wilson and Jones, 1983) but the significance of this is not known. Loss of DNA methylation or changes in methylation patterns could explain the aberrant gene expression observed in aged cells. Although Romanov and Vanyushin (1981) detected a reduction in the level of DNA methylation between embryonic cows and old cows, Ehrlich *et al* (1982) failed to show an overall decrease in the level of DNA methylation with age in vivo. Accelerated loss of methylation in fibroblasts results in a reduced lifespan of the cells *in vitro* (Fairweather et al., 1987).

1.3.4 Aging and the heat shock proteins

Increased levels of basal HSP expression with age

The presence of abnormal proteins can induce the heat shock response (Goff and Goldberg, 1985; Ananthan et al., 1986). Since it has been shown that there is accumulation of damaged proteins in ageing cells, an increase in the levels of heat shock proteins would be predicted in unstressed aged cells and in fact this has been shown to

be correct. In senescent human fibroblasts, basal levels of HSP 27, HSC 70 and HSP 70 are all increased in late passage cells (Fonager et al., 2002). In the absence of stress there is increased transcription of some of the heat shock response proteins, including HSP 27 and HSP 70, in old mouse muscle cells (Lee et al., 1999). An age associated increase in HSP 70 expression is also observed in *Drosophila melanogaster* but appears to be posttranslational and specific to the flight and leg muscles (Wheeler et al., 1995).

Diminished response to acute stress with age

Although there is an increase in the basal levels of heat shock proteins in aged cells, the induction of the heat shock proteins in response to acute stress is diminished with age. In replicatively senescent fibroblasts the induced levels of mRNA and protein for HSP 70 and HSP 90 are reduced after heat shock and exposure to sodium arsenite, as is the level of HSP 27 mRNA. A reduction in the total level of HSP 60 and HSP 90 expression after heat shock is also observed in old compared to young fibroblasts (Liu et al., 1989a; Liu et al., 1989b; Luce and Cristofalo, 1992).

After heat stress lower levels of HSP 70, but not HSP 27, are observed in fibroblasts from old animals than in fibroblasts from young animals at both the mRNA and protein levels (Fargnoli et al., 1990). The same reduction in HSP 70 levels after stress is reported at the mRNA level in human peripheral blood mononuclear cells and at the protein level in Rhesus monkeys, as well as in splenic lymphocytes from rats (Deguchi et al., 1988; Pahlavani et al., 1995). Decreased induction of the proteins HSP 90 and HSC 70 following mitogen stimulation is observed in lymphocytes from aged donors compared to young donors (Faassen et al., 1989).

Heat shock induces both HSP 70 and HSC 70 in young and old hepatocytes taken from rats, but the induction is about 30% lower in the old cells (Wu et al., 1993). Also in hepatocytes Heydari et al (1993) show a decline in HSP 70 levels with age after heat shock. The observed decline in induction of HSP 70 is not due to an accumulation of cells that could not express HSP 70 or a decline in the stability of the mRNA but there is a decline in transcription of HSP 70. Even in skin, where stress response mechanisms are very important and are in regular use, organ cultures reveal that the induction of HSP 70 by heat shock is impaired with age (Muramatsu et al., 1996).

An age related decline in the heat shock response is also observed *in vivo*. The induction of HSP 70 in the adrenal and vascular tissues in response to restraint stress in rats is reduced with age (Blake et al., 1991b; Udelsman et al., 1993; Fawcett et al., 1994). The ACTH and corticosterone levels are elevated to a similar degree in both old and young animals undergoing restraint stress, suggesting that the degree of stress experienced by the rats is similar and that the HPA is not impaired in old age (Blake et al., 1991b; Udelsman et al., 1993). Only one report of an age related decline in heat shock protein induction by stress has reported a corresponding decrease in the degree of stress experienced by the animals (Blake et al., 1991a). An attenuation of the induction of HSP 70 in rat hearts response to hyperthermia and exercise is also observed (Demirel et al., 2003), as is reduced expression of HSP 70 and HSC70 in the hearts of old rats in response to ischaemia (Nitta et al., 1994).

Aged rats show a marked reduction in the levels of HSP 70 in their livers after heat shock. Young animals show some induction of HSC 70 and a substantial induction of HSP 70 for 48hrs. The senescent animals show a similar level of induction of HSC 70, i.e. very slight, and a substantial induction of HSP 70 2 hrs after stress but this induction is not sustained. The older rats suffered zone specific liver injury and reduced thermotolerance (Hall et al., 2000).

Possible mechanism for the loss of heat shock protein induction

HSF-1 increases expression of the heat shock proteins following a variety of stresses including; restraint, thermal stress, exposure to heavy metals and exposure to amino acid analogues, by trimerizing and translocating to the nucleus where it binds to the heat shock element in the genes that are induced as part of the heat shock response. Although the induction of HSP 70 in the adrenal and vascular tissues in response to restraint stress is reduced with age, the level of HSF-1 in the tissues is not reduced. The level of HSE-binding by HSF-1 however is reduced in old age both in the presence and absence of stress. Fawcett et al (1994) also report reduced trimerization of HSF-1 in aged rats. Interestingly the level of HSE-binding was directly additive when extracts from the cells of old and young animals were combined. The authors suggest that this would not be case if there was a DNA binding inhibitor or activator involved (Fawcett et al., 1994).

An age associated decrease in the DNA binding activity of the heat shock transcription factor was also found *in vitro* in the fibroblast cell line IMR-90 (Choi et al., 1990), and in human and rat lymphocytes (Pahlavani et al., 1995; Jurivich et al., 1997). Interestingly Choi *et al* (1990) reported that when the extracts from young and old cells were combined the DNA binding activity decreased more than would be expected with the addition of extract from old cells. Jurivich *et al* (1997) also present some evidence for the presence of a DNA binding inhibitor in the aged cells but it is far from unequivocal. These studies were carried out *in vitro* and this may explain the difference between them and the Fawcett et al (1994) paper in which data from an *in vivo* study using a physiological stress is presented. It should be noted that Demirel et al (2003) also fail to show decreased HSF-1 binding to HSE in aged animals in response to hyperthermia and exercise.

Manalo et al. (2002) have proposed that disulphide cross linking is dependent upon the redox environment in the cell and acts to prevent inappropriate activation of HSF-1. Since oxidative damage appears to be a key aspect of ageing they suggest that oxidation could cause intramolecular disulphide cross-linking and could therefore contribute to the age related attenuation of HSF-1 activity.

Since the activity of HSF-1 declines with age this is a possible explanation for the observed decline in the heat shock response with age, although more work is obviously needed to understand how and why the activity of HSF-1 becomes reduced. The evidence that the HSP stress response becomes impaired in old age is very strong, since the impairment has been described in cell culture, in organ culture and in vivo. The system appears to fail at a number of places in the stress response pathway, the binding of HSF-1 to the HSE, the transcription of the heat shock proteins, the translation of the heat shock proteins (Lund et al., 2000; Visala Rao et al., 2003) and the degradation of damaged proteins.

There are a number of ways to induce or increase heat shock protein expression in young cells. Two peptides known to induce heat shock proteins in young cardiac myocytes are CT-1 and UCN and these were used in the present study to try to increase

endogenous heat shock protein expression in sensory neurons and to investigate any protective effect in older cells.

1.4 Cardiotrophin – 1

Cardiotrophin – 1 (CT-1) is a 21.5KDa protein which was initially isolated due to it's ability to induce hypertrophy in rat neonatal cardiac myocytes *in vitro* and was cloned from a mouse embryoid body cDNA library (Pennica et al., 1995a). The mouse CT-1 gene contains a variety of transcription factor binding motifs including GATA, Nuclear factor for IL-6 (NF-IL-6), p53 and Hypoxia inducible factor (HIF-1) (Funamoto et al., 2000). Based on the primary sequence and the predicted tertiary structure it is part of a family of cytokines that includes Leukemia inhibitory factor (LIF), Ciliary neurotrophic factor (CNTF), Oncostatin M (OSM), interleukin – 6 (IL-6) and interleukin – 11 (IL-11).

1.4.1 The IL-6 family of cytokines

IL-6 is a pleiotrophic cytokine with functions in the immune system as well as the hematopoietic, endocrine, hepatic and neural systems (Kishimoto et al., 1994). CNTF is a neurotrophic factor that maintains survival of ciliary neurons and induces differentiation of sympathetic neurons (Adler et al., 1979; Ernsberger et al., 1989; Saadat et al., 1989). CNTF also has a trophic effect on DRG neurons *in vitro* (Simon et al., 1995). LIF acts as a differentiation factor, switching the phenotype of postmitotic sympathetic neurons from noradrenergic to cholinergic (Yamamori et al., 1989). The *in vitro* activities of oncostatin M include: inhibition of the growth of solid tumor cells (Horn et al., 1990), induction of differentiation of leukemia cells (Rose and Bruce, 1991), stimulation of IL-6 production in Kaposi's sarcoma cells and human endothelial cells (Brown et al., 1991; Miles et al., 1992), stimulation of the production of a urokinase-type plasminogen activator in synovial fibroblast-like cells (Hamilton et al., 1991) and induction of acute phase protein synthesis in HepG2 cells and rat hepatocyte cultures (Richards et al., 1992). These *in vitro* activities suggest potential *in vivo* roles in hematopoiesis and inflammation (Bruce et al., 1992). *In vitro* IL-11 stimulates: erythropoiesis (Quesniaux et al., 1992) and hepatic acute phase response (Baumann and

Schendel, 1991), *in vivo* and *in vitro* IL-11 modulates the antigen specific antibody response (Yin et al., 1992).

CT-1 is 24% identical to LIF and 19% identical to CNTF, it is predicted to have four amphipathic α helices like the other members of the family and like CNTF it does not have an N-terminal secretion signal sequence (Stockli et al., 1989; Pennica et al., 1995a). Genomic clones of human CT-1 encode a 201 amino acid protein that is 80% identical to the mouse protein (Pennica et al., 1996b). Another member of the family has also been identified, Cardiotrophin-like cytokine (CLC) has a 29% identity to CT-1 and the corresponding mRNA is expressed in human spleen and peripheral leukocytes. Like other members of the family CLC induces the phosphorylation of gp130 and STAT-1 (Shi et al., 1999).

1.4.2 The receptors

The receptors for the IL-6 family of cytokines are made up of multisubunit complexes and all include the glycoprotein 130 (gp130) (Taga et al., 1989; Pennica et al., 1995a; Pennica et al., 1995b). The receptors are characterized by four positionally conserved cysteine residues and the WSXWS motif (Taga and Kishimoto, 1997). IL-6 and IL-11 induce homodimerization of the gp130 subunit (Murakami et al., 1993) whereas CT-1, LIF, OSM and CNTF induce heterodimerization of the gp130 subunit with the glycoprotein 190 (gp190) subunit, gp190 is also known as LIFR (Davis et al., 1993; Pennica et al., 1995b). Gp130 is able to cross react with different ligands because it has thermodynamic plasticity that allows it to be relatively insensitive to ligand structure (Boulanger et al., 2003). All of the receptors also require a third component often referred to as the α component, which differs for each member of the family although there is evidence that CNTF can also utilize the IL-6R (Yamasaki et al., 1988; Heymann et al., 1996; Schuster et al., 2003). CT-1Rα, is an 80KDa component isolated from the human neuroblastoma cell line SK-N-MC (Robledo et al., 1997). CT-1Rα forms a tripartite receptor with gp130 and LIFR whereas IL-6R, the α component for IL-6, forms a tripartite receptor with two gp130 subunits. The receptors appear to be highly conserved because there is a high level of homology in the receptor system (Taga and Kishimoto, 1997). LIFR lacks species specificity, since human and mouse CT-1 bind to

LIFR in both human and mouse cell lines (Pennica et al., 1996b). The IL-6 gene is also highly conserved with 60% similarity between the mouse and human coding sequence and up to 80% similarity between the 3' untranslated region and the first 300bp of the 5' untranslated region (Tanabe et al., 1988).

IL-6R and gp130 are also found as soluble receptors. The physiological roles of these soluble receptors is not clear but it has been proposed that they act as buffers to modulate the systemic effects of IL-6 (Heinrich et al., 1998). See Heinrich et al. (1998) for a thorough review of the IL-6 family of cytokines, their receptors and the relevant signalling pathways.

1.4.3 Signalling

Activation of the IL-6 cytokine receptors causes activation of the JAK/STAT pathway (Darnell, Jr. et al., 1994) including activation of the tyrosine kinases Jak1, Jak2 and Tyk2 (Stahl et al., 1994; Narazaki et al., 1994; Lutticken et al., 1994; Yin et al., 1994) and the transcription factors STAT1 and STAT3 (Sadowski et al., 1993; Zhong et al., 1994; Darnell, Jr. et al., 1994). Shc, Grb2 and SOS are recruited leading to the activation of Ras and therefore activation of the Raf/MEK/ERK pathway, the PI-3Kinase/AKT pathway and the p38MAPK pathway, which all result in the activation of the transcription factor NF-κB (Craig et al., 2001).

STAT-1 and STAT-3 are recruited to phosphotyrosine modules in the cytoplasmic domain of gp130, STAT-1 binds to the consensus sequence YXPQ and STAT-3 binds to the consensus sequence YXXQ (Stahl et al., 1995; Gerhartz et al., 1996). The specificity of binding is determined by the SH2 domain of both proteins (Heim et al., 1995; Hemmann et al., 1996).

Once activated, STAT-1 and STAT-3 are translocated to the nucleus where STAT-1 enters the nucleus by the conventional import pathway mediated by importin (NPI-1 and the β subunit of the nuclear pore targeting complex) (Sekimoto et al., 1997). STAT-3 appears to form a complex with the glucocorticoid receptor that contains two nuclear localization signals (Picard and Yamamoto, 1987; Zhang et al., 1997). Once in the

nucleus the STATs bind to the consensus sequence $TTCN_{(2-4)}GAAA$ (Seidel et al., 1995). A diverse range of genes are activated by IL-6 cytokine mediated STAT signalling, some are acute phase proteins such as lipopolysaccharide binding protein and tissue inhibitor of metalloproteinases (TIMP): Others are transcription factors such as JunB and the CCAAT enhancer binding protein delta; Bcl-x is also activated as are HSP 90 and gp130. The upregulation of gp130 expression by a STAT and the IL-6 cytokine family suggests a positive feedback loop (Coffer et al., 1995; Bugno et al., 1995; Schumann et al., 1996; Yamada et al., 1997; Stephanou et al., 1997; O'Brien and Manolagas, 1997; Fujio et al., 1997; Stephanou et al., 1998b).

After transient activation the signalling pathway is inactivated by several different methods. These include the protein inhibitors of activated STAT-3 and STAT-1 (PIAS3 and PIAS1) which specifically inhibit the corresponding STAT protein, the cytokine inducible SH-2-containing protein family (CIS), also known as suppressor of cytokine signalling (SOCs), or STAT induced STAT inhibitors (SSIs) or Jak binding proteins (JAB). Dephosphorylation and proteolytic degradation can also deactivate the cytokine mediated activation of the STAT proteins (Haspel et al., 1996; Kim and Maniatis, 1996; Naka et al., 1997; Endo et al., 1997; Chung et al., 1997; Starr et al., 1997). LIFR is downregulated by ERK, since ERK phosphorylates serine 185 of the LIF receptor cytoplasmic domain which promotes the endosomal/lysosomal pathway of degradation and therefore reduces the half life of LIFR (Blanchard et al., 2000). This appears to be a negative feedback control mechanism since LIF as well as OSM and insulin activate ERK.

1.4.4 CT-1 and the heart

CT-1 appears to have an important role in the heart during and after development. It is first expressed in the heart at embryonic day 8.5 in mice and promotes survival of the cardiac myocytes (Sheng et al., 1996). CT-1 is expressed abundantly in the heart and the cAMP response element (CRE) provides a potential cis-acting element to activate transcription of CT-1 in response to norepinephrine (Funamoto et al., 2000). CT-1 has been detected in the ventricles of genetically hypertensive rats (Ishikawa et al., 1996), and is increased during ventricular remodeling in hypertensive rats (Takimoto et al.,

2002). CT-1 is elevated in heart failure (Jougasaki et al., 2000; Aoyama et al., 2000), this may be an attempt to overcome diminishing levels of gp130 protein (Zolk et al., 2002). Although it lacks a conventional secretion signal CT-1 is secreted by the heart (Talwar et al., 1999; Asai et al., 2000; Talwar et al., 2000; Talwar et al., 2001; Ng et al., 2002; Talwar et al., 2002). CT-1 protects cardiac myocytes from thermal and ischaemic stresses and serum withdrawl (Stephanou et al., 1998a; Brar et al., 2001a) and myocardial muscle sections against ischaemia (Ghosh et al., 2000). CT-1 activates the extracellular regulated kinase (ERK), MAPK and Akt pathways in cardiac myocytes (Sheng et al., 1997; Brar et al., 2001b). Craig et al. (2001) show that the protective effect of CT-1 in cardiac myocytes is not only dependent on the activation of these pathways but also requires activation of NF-κB. CT-1 causes hypertrophy through a different pathway from the p42/44 MAPK pathway through which it has a protective effect. HSP 56 is induced by CT-1 in cardiac myocytes and mediates the hypertrophic effect via STAT3 (Latchman, 2000; Railson et al., 2001; Railson et al., 2002). HSP 70 is induced by CT-1 in cardiac myocytes and HSP 90 is induced by CT-1 in both cardiac myocytes and the cardiac cell line CLEM, see 3.1 for further details (Stephanou et al., 1998a; Railson et al., 2000).

1.4.5 Other functions of CT-1

In mice CT-1 causes a dose dependent increase in heart weight and ventricular weight but not an increase in body weight indicating that CT-1 causes hypertrophy *in vivo*. It also increases growth of the liver, kidneys and spleen as well as increasing platelet counts and red blood cell counts (Jin et al., 1996). CT-1 mRNA is expressed in a range of tissues including heart, skeletal muscle, ovary, colon, prostate and testis in adult humans and in fetal lung and kidney (Pennica et al., 1996b). The observed increases in LIF, IL-6, CNTFRα, LIFRβ and IL-6Rα in peripheral neuropathies and the increases in IL-11 and OSM after nerve injury suggest that the IL-6 family of cytokines could be involved in the repair and regeneration of neurons (Curtis et al., 1994; Hirota et al., 1996; Tham et al., 1997; Ito et al., 2000; Ito et al., 2001). The IL-6 cytokines also promote survival of embryonic peripheral neurons and, as in the heart, the protective effect is dependent on NF-κB activation (Horton et al., 1998; Middleton et al., 2000).

1.5 Urocortin

Urocortin (UCN) is a neuropeptide related to corticotropin releasing factor (CRF) and urotensin. UCN has a 45% sequence identity with CRF and a 63% identity with urotensin. CRF is a peptide that regulates the hypothalamic-pituitary-adrenal axis and endocrine and behavioural responses to stress (Vale et al., 1981; Vaughan et al., 1995). The mouse UCN gene encodes a 122 amino acid precursor protein and a 40 amino acid mature peptide. The precursor protein is 95% identical to the rat amino acid sequence and the mature peptide is 100% identical to the rat peptide at the amino acid level. UCN is a highly conserved peptide as the human UCN peptide is also 95% identical to the rat sequence at the amino acid level. The promoter region contains a cyclic AMP response element, GATA binding sites, a TATA box and several brn-2 binding sites in both the mouse and human gene (Donaldson et al., 1996; Zhao et al., 1998). Other members of the family include UCN II and UCN III (Reyes et al., 2001; Hsu and Hsueh, 2001; Lewis et al., 2001)

1.5.1 Urocortin expression

Although there have been several studies of UCN expression none has been as thorough or systematic as Bittencourt et al (1999). The immunoreactivity they describe agrees with the original study of Vaughan et al (1995). Urocortin mRNA and peptide are expressed in the Edwinger-westphal (EW), lateral superior olivary (LSO) and supraoptic nuclei. In the hypothalamus they also describe some immunoreactive cells in the paraventricular nucleus. Lower levels of expression are also detected in some cranial nerves, some spinal motor neurons and some forebrain neurons. In the peripheral tissues there is immunoreactivity in the gut, stomach, duodenum and lymphoid tissue. They did not detect urocortin immunoreactivity (UCN-ir) in the pancreas, kidneys, adrenal gland, heart or testis.

There are UCN-ir projections in the midbrain from the EW to the red nucleus, substantia nigra and the periaqueductal gray, as well as the dorsal raphe nucleus, ventral tegmental nucleus, basilar pontine nuclei and parabrachial nuclear complex. From the LSO there are fibres to the vestibular and cochlear nuclei, medial superior olive, trapezoid body, lateral lemniscus and inferior colliculus. There are some fibres

projecting to the cerebellar cortex and medulla. In the medulla there are projections from the EW to facial, lateral reticular, spinal trigeminal nuclei and the inferior olivary complex.

In the forebrain there are UCN-ir fibres in the rostral periaqueductal gray, the posterior hypothalamic area and the nucleus reunions of the thalamus. Apart from fibres in the hypothalamus and limbic forebrain, staining in the forebrain is sparse and diffusely organized. There are immunoreactive fibres in the optic tract and very sparse staining in the cerebral cortex, basal ganglia, amygdala and hippocampus. There are moderately immunoreactive projections to the posterior pituitary.

In the spinal cord fibres project to the spinal gray in a similar manner at all levels. There are also some fibres in the ventral horn including a small number of fibres directly apposed to motor neurons (Bittencourt et al., 1999).

1.5.2 The urocortin receptors

UCN binds to the corticotropin releasing factor receptors 1 and 2. CRFR1 was originally isolated from the brain and is expressed in the pituitary, cerebellum, cerebral cortex and olfactory bulb, it is also expressed in the spleen and thymus. CRFR2 was isolated from the brain and heart and it has also been identified in the spleen and thymus (Chang et al., 1993; Vita et al., 1993; Perrin et al., 1993; Chen et al., 1993; Kishimoto et al., 1995; Stenzel et al., 1995; Vaughan et al., 1995; Donaldson et al., 1996; Baigent and Lowry, 2000a; Baigent and Lowry, 2000b). There are two splice variants of CRFR2, CRFR2α is expressed in some areas of the brain including the lateral septum, ventromedial nuclei of the hypothalamus and the amygdala (Lovenberg et al., 1995b) whereas CRFR2β is expressed in the hypothalamus and hippocampus in the brain and peripheral tissues including the duodenum, skeletal muscle, lung and the heart (Perrin et al., 1995; Lovenberg et al., 1995a). A third form of the CRFR2 receptor has been described but so far CRFR2γ has only been detected in human brain (Kostich et al., 1998).

1.5.3 Functional activity of urocortin

UCN is expressed by human pituitary cells; it is anxiogenic causing decreased social interaction and decreased exploration in maze tests when delivered centrally but is not as potent in this regard as CRF (Iino et al., 1997; Moreau et al., 1997; Sajdyk et al., 1999; Skelton et al., 2000). It also stimulates ACTH release from pituitary cells *in vitro* (Vaughan et al., 1995; Donaldson et al., 1996; Asaba et al., 1998; Ozawa et al., 1998). UCN and CRFR2 knockout mice both display increased anxiety like behaviour, CRFR2 knockout mice display increased responses to acute stress but the UCN knockout mice do not have altered stress hormone secretion profiles after acute stress. UCN knockout mice also have impaired hearing. CRFR2 knockout mice have an impaired cardiovascular response to UCN, normal basal feeding but decreased food intake after food deprivation and there is no indication of impaired hearing in these mice (Coste et al., 2000; Bale et al., 2000; Vetter et al., 2002). CRFR1 knockout mice have impaired stress responses and display decreased anxiety like behaviour. They have low levels of plasma corticosterone because the adrenal gland does not develop correctly, due to insufficient adrenocorticotropic hormone (ACTH). The CRFR1/CRFR2 double knockout also has an impaired stress hormone response and atrophied adrenal glands suggesting that CRFR1 has a crucial role in the development of the hypothalamic-pituitary-adrenal axis and in mediating stress responses (Smith et al., 1998; Timpl et al., 1998; Zorrilla et al., 2002; Bale et al., 2002). It appears that UCN has a far greater affect on peripheral tissues, motor function and sensorimotor integration, and CRF is a more important ligand in the CNS mediating stress induced and anxiety related behaviours and the endocrine responses to stress through CRFR1.

UCN is expressed in the enteric nervous system, including the duodenum, the small instestine and the colon (Harada et al., 1999; Bittencourt et al., 1999), and inhibits gastric emptying and food intake through the receptor CRFR2 with greater potency than CRF (Spina et al., 1996; Asakawa et al., 1999; Nozu et al., 1999; Bradbury et al., 2000). A role has been suggested for UCN in the regulation of metabolism (Kotz et al., 2002; De Fanti and Martinez, 2002). UCN is expressed by the placenta during pregnancy (Petraglia et al., 1996). UCN is also expressed in the synovial tissue of patients with rheumatoid arthritis, suggesting that it has a role in inflammatory responses (Uzuki et al., 2001; Kohno et al., 2001).

In the heart UCN but not CRF induces a dose dependent increase in heart rate, cardiac output and coronary blood flow (Parkes et al., 1997). UCN also induces HSP 90 expression in cardiac myocytes and protects them through the same signalling pathway as CT-1, UCN has also been shown to increase CT-1 expression in cardiac myocytes (Railson et al., 2002; Brar et al., 2002a; Janjua et al., 2003).

Another method of increasing heat shock protein expression is using exogenous expression by delivering the heat shock protein genes, under strong promoters, to the cells.

1.6 Viral vectors

There are many different ways to deliver transgenes to cells. A virus vector was used in this thesis because none of the classical transfection methods are very efficient in mature neurons (Washbourne and McAllister, 2002). There are two main types of viral vector currently used, herpes viral vectors and adenoviral vectors, and there are pros and cons for each. Adenoviral vectors are often chosen because it is easier to grow higher titre stocks of adenoviruses and the vector can be used to deliver fairly large inserts. However, the efficiency of infection in postmitotic neurons is not as high as herpes viral vectors (Ehrengruber et al., 2001), and a longer period of time is required for expression to begin (Vasquez et al., 2001). Gutless adenoviral vectors are being developed and whilst they may reduce or eliminate the immune reactions sometimes caused by adenoviral vectors (Bhat and Fan, 2002) and transduce neurons more efficiently, they require helper viruses and are technically difficult to work with. Adeno-associated viruses are less toxic than either the adenoviral vectors or the herpes viral vectors, they give a high level of expression and efficiently infect postmitotic neurons (Kaplitt et al., 1994; Peel and Klein, 2000). Adeno-associated viral vectors are not the vector of choice because they can only deliver up to 5Kb inserts and it takes a period of about two weeks for expression to begin (Ehrengruber et al., 2001). The herpes virus vectors can be problematic *in vivo* because of their toxicity and lack of long term expression (Feldman, 1996). However, they can be used to deliver very large inserts and they infect

postmitotic neurons efficiently giving a high level of expression quickly, making them an almost ideal vector for this work (Geller and Breakefield, 1988; Simonato et al., 2000; Latchman, 2001; Washbourne and McAllister, 2002).

1.6.1 Herpes simplex virus vectors

Herpes simplex virus (HSV-1) is a double stranded DNA virus that can either enter a lytic cycle or persist in a latent state as an episome (Rock and Fraser, 1983; Rock and Fraser, 1985). HSV-1 viral vectors have key viral genes deleted so that the virus cannot enter the lytic cycle and replicate. The herpes virus vector used in this thesis, WΔMN+, had the immediate early gene ICP27 deleted to prevent trans-activation of the early and late viral genes. This deletion not only prevents the vector from being replication competent but also reduces the toxicity because it is the immediate early genes which are responsible for the cytopathic effects of the virus (Johnson et al., 1994). Expression of the transgene is under the control of the cytomegalovirus immediate early promoter (CMV-IE). This is a strong promoter and was used in combination with the latency associated promoters to provide maximum expression of the transgene (Wagstaff, 1997).

1.7 Aims

The endogenous heat shock response is impaired with age and there are also many other age related changes that occur at the cellular and molecular level. The block in the heat shock response appears to be due to a loss of DNA binding activity by HSF-1 which therefore fails to initiate heat shock protein expression. It is not known whether, if this impairment could be overcome, heat shock proteins would be sufficient to protect aged cells against stressful conditions since there are so many other age related changes. Also a non-harmful way of inducing heat shock protein expression in neurons could be useful because the loss of neurons in conditions such as stroke and neurodegenerative disease is very debilitating, for instance after a stroke there are two periods of cell death, initially the cells in the centre of the hypoxic area die and later a second wave of cell death occurs around the hypoxic area (Barinaga, 1998). If the second wave of cell death

could be prevented perhaps the loss of function often resulting from a stroke could be greatly reduced. CT-1 and UCN have both been shown to induce endogenous heat shock protein expression and to protect cardiac myocytes against physiological stresses such as simulated ischaemia.

Therefore this study set out to examine whether CT-1 and UCN could be used to induce heat shock protein expression in sensory neurons and to test whether they could protect sensory neurons against stressful conditions such as heat stress and hypoxic ischaemia. Viral vectors containing HSP 27, HSP 70 or HSP 56 were used to test whether increasing levels of heat shock protein expression to compensate for the age related decline would be sufficient to protect aged sensory neurons against lethal heat and ischaemic stresses.

Chapter 2: Materials and methods

2.1 Chemicals

Laboratory chemicals were purchased from Sigma Chemical Company Ltd, Poole, Dorset, UK unless otherwise stated. Reagents were also purchased from Roche Diagnostics Ltd, Lewes, East Sussex; Invitrogen, Paisley, Scotland; Insight Biotech, Wembley, London and Promega, Promega UK, Southampton. Microscopes were from Zeiss, Welwn Garden City, Herts, UK.

Tissue culture plastics were purchased from Nalge Nunc International, Nalge Europe Ltd, Hereford, UK. Roller bottles were purchased from Corning, Gordon Road, High Wycombe, Bucks., UK. F14 powdered media was purchased from Imperial, Andover, Hampshire, UK. Collagenase and Trypsin were purchased from Worthington Biochemical corporation, Lakewood, New Jersey, USA.

Oligonucleotide primers were purchased from Thermoelectron, Ulm, Germany. Hybond-C nitrocellulose membranes and Rainbow protein markers were purchased from Amersham Bioscience UK Ltd, Little Chalfont, Amersham, Bucks. UK. Broad range protein markers were purchased from New England Biolabs (UK) Ltd, Hitchin, Herts, UK.

All chemicals were dissolved in ddH_2O and adjusted to the required pH using HCl or NaOH, and autoclaved or filter sterilized unless otherwise stated.

2.2.1 Common Solutions

PBS

PBS tablets (Invitrogen):

0.14M NaCl

0.01M PO_4 Buffer

0.003M KCl

pH 7.45

TAE

40mM Tris acetate

2mM EDTA

Approximately pH8.3

2.2 Tissue Culture

All incubations were carried out in an incubator at 37°C, 5% CO_2 and 95% humidity. All dissections were carried out in a laminar flow hood (with a 0.2μM filter) using standard sterile technique, subsequent preparation of the neuronal cultures and all other tissue culture work was carried out in a class 2 cabinet using standard sterile technique. A stereomicroscope with fibre-optic lights was used for the dissections. The sensory neurons were plated on coverslips coated with poly-D-ornithine (0.5mg/ml) overnight at 37°C, 5% CO_2 and 95% humidity, washed twice with ddH_2O, dried and then coated with 40μl droplets of 20μg/ml laminin for at least two hours before use. The laminin was removed and the coverslips were washed with 40μl of media, see 2.2.1, before the cells were plated.

2.2.1 Media

Experiments were either carried out in defined media (Davies, 1995) or DMEM with phenol red and glutamine, 0.11g/l sodium pyruvate and pyrodoxine (Invitrogen) supplemented with 10% (v/v) foetal calf serum (Insight Biotech).

Defined media
10% (v/v) F14 10x (Imperial labs)
84% (v/v) ddH_20
0.2% (w/v) $NaHCO_3$
2mM L-glutamine (Invitrogen)
0.375% Bovine serum albumin (Fraction V)
60ng/ml progesterone
16ug/ml putrescine
400ng/ml L-Thyroxine
38ng/ml sodium selenite
340ng/ml Tri-iodo-thyronine (T3)
10ng/ml NGF (Invitrogen) for neonatal cultures only
1% penicillin streptomycin
pH7
10% Foetal calf serum (Insight Biotech) was used to supplement adult cultures

Table 2.1: The constituents of the defined media and their concentrations.

Where serum free media is stated the media was as above but did not contain bovine serum albumin or foetal calf serum.

2.2.2 Primary culture of sensory neurons.

Neonatal Sprague Dawley were culled by cervical dislocation at postnatal day one. Adult Sprague Dawley were 150g, approximately 6 weeks old and aged Sprague dawley were 18 – 23 months old, approximately 800g - 1kg. Adult and aged rats were culled using a slowly rising concentration of CO_2, gas flow was set at 4l/min and increased to

6l/min once the animal was unconscious. The aged rats were purchased at 12 months old and kept for at least 6 months until they were old enough to use. The spinal column was removed from the body by removing the skin from the back, then cutting through the spine at the top and bottom and then cutting through the pelvis and ribs on either side. Any excess flesh was removed from around the spine to expose the vertebrae. Cutting from the top down the vertebrae were cut about half way around the circumference on each side and the dorsal half of the spinal column was removed. The spinal cord was then removed to expose the dorsal root ganglia. Using forceps and small curved scissors each dorsal root ganglia was removed from between the vertebrae and placed in Hanks balanced salt solution without calcium or magnesium and without phenol red (HBSS)(Gibco). The spinal nerve and posterior dorsal root were removed along with any extraneous material. To dissociate the cells the neonatal ganglia were placed in 0.3% type 2 collagenase (Worthington Biochemical corporation) and 0.1% trypsin (Worthington Biochemical corporation) in HBSS and incubated at 37°C/5% CO_2/95% humidity for 20 minutes. Adult and aged ganglia were placed in 0.3% type 2 collagenase in HBSS and incubated for 1 hour. After incubation the ganglia were triturated to a single cell suspension.

It was desirable to remove as many non-neuronal cells as possible from the culture since they can synthesize and release neurotrophic factors in culture and therefore allow indirect effects on the neuronal cells. Differential adhesion and differential sedimentation can both be used to remove non-neuronal cells. Differential sedimentation was the chosen method as this was considered to be more effective and reliable, it removes the problem of cell aggregation, which can occur in differential adhesion, and permits high neuronal viability. The cells were spun in a Mistral 1000 centrifuge at 900 rpm for 5 minutes through a 6% metrizamide cushion, to remove most of the non-neuronal cells, method modified from (Camu and Henderson, 1992).

To prevent the division of any remaining contaminating glial cells, cells cultured in DMEM were treated with cytosine β-D-arabinofuranoside (AraC) at a final concentration of 10μM, this concentration has previously been used in cultures of neonatal and adult sensory neurons (Lindsay, 1988; Mulderry, 1994). AraC blocks mitosis by inhibiting DNA synthesis, RNA synthesis is unaffected by it. It was not

necessary to add AraC to cultures in F14 as this is a defined media and so does not support the growth of non-neuronal cells to the same extent. AraC was not used in cultures infected with the virus vectors because the combination caused some toxicity.

Neurons were plated at a density of approximately 1000 – 2000 cells per well. Due to the low neuron density required the most reliable way to estimate the density was by eye (Davies, 1995).

2.3 Herpes simplex virus vector

A herpes simplex virus vector (WΔMN+) derived from the Glasgow 17+ strain was used to introduce the heat shock protein genes for HSP 27, HSP 56, HSP 70 and green fluorescent protein (GFP) into dorsal root ganglia cells. Figure 2.1 shows the structure of the virus vector used.

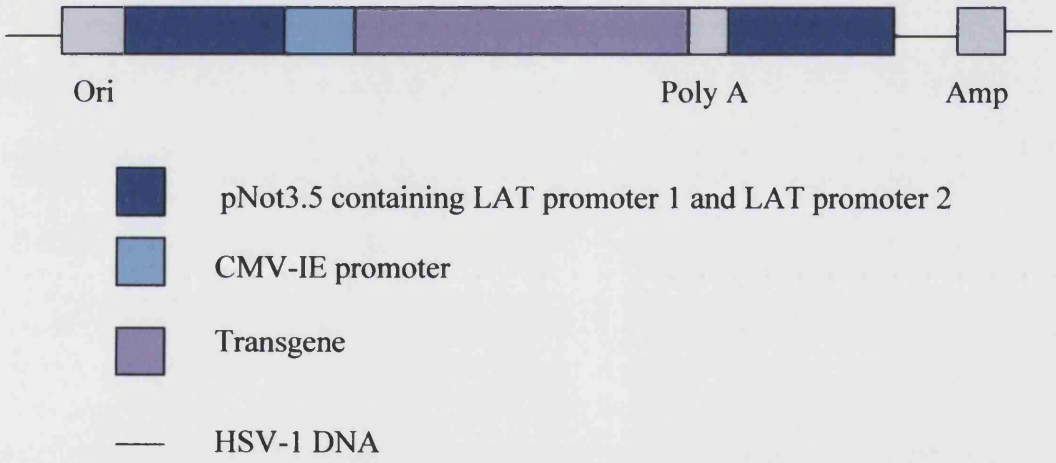


Figure 2.1 Map of the virus construct used. Virus construct 17+pR19 was made by Marcus Wagstaff (Wagstaff, 1997) The HSP cDNAs used in the virus construction are Chinese hamster HSP 27, rabbit HSP 56 and human inducible HSP 70. CMV-IE is the CMV immediate early promoter, pNot3.5 is a plasmid containing the latency associated transcript (LAT) regions of the HSV-1 genome.

The HSV-1 virus vectors used were ICP 17+/27- so the complementing cell line MAM49 had to be used to produce stocks of the virus. MAM49 is a stably transfected line of baby hamster kidney (BHK) cells that contains two viral genes, ICP 4 and ICP 27 both of which are necessary for replication of the viruses although the virus vector used is only lacking ICP 27. The complementing cell lines allow the virus to replicate and enter the lytic phase by producing the proteins that correspond to the genes deleted in the virus vector.

2.3.1 Expanding Virus stocks

The cell line MAM49 was grown under selection to maintain the prescence of the plasmids in the complementing cell line. Geneticin (G418) and Zeocin were used at a concentration of 0.64mg/ml and 0.07mg/ml respectively, the concentrations were determined by kill curves previously carried out in this laboratory.

The cells were split when they were confluent using 0.25% trypsin in versene 1:5000 (Invitrogen). The media was removed and the cells washed once with HBSS. After a brief incubation in 0.25% trypsin the cells were physically dislodged from the flask and selection media was added. The cells were then used to seed new flasks in selection media. Full growth media consisted of DMEM plus 10% foetal calf serum, selection media also contained G418 and zeocin.

A 6 well plate of cells was infected in serum free media. These cells were left for 1 hour and then full growth media was added with N,N'– hexamethylenebisacetamide, 98% (HMBA) (Aldrich chemical company, Inc) at 56ng/ml. HMBA has been shown to stimulate expression of the viral immediate early genes in the absence of VMW65 (Ace et al., 1988; Estridge et al., 1990; McFarlane et al., 1992), although the pR19 virus vectors do not lack VMW65 any increased expression of the immediate early genes may increase the titre of the virus vector produced. After 48 hours the infected cells have lysed to release the virus particles into the media. The media from the infected, lysed cells was then used to infect more cells, 3mls were used to infect a confluent large flask

and 25mls were used to infect a roller bottle. Tissue culture roller bottles have an expanded surface area of 1700cm^2 (Corning) and were turned at 1rpm.

A minimum of 500mls of media from infected cells was collected before purifying the virus. The plates were scraped and the media frozen at -80°C and stored until required or until the media was fully frozen if required straight away. When required the media was thawed at room temperature and spun for 45 mins at 3500 rpm. The supernatant was then filtered through a 0.22μM filter and placed in autoclaved beckman centrifuge tubes. These were then spun for 2 hours at 12 000 rpm in a sorvall R26 centrifuge. The supernatant was discarded and the pellets were resuspended in 750μl - 1000μl of serum free media (DMEM), divided into 20μl aliquots and stored at –80°C.

To titre the virus, MAM49 cells were plated in a 6 well plate at approximately 90% confluency. A serial dilution of the virus was prepared from 10^{-4} to 10^{-10} in serum free DMEM and added to the cells. After 1 hour twice the volume of 1:1 carboxymethyl cellulose:Full growth media plus HMBA was added and the cells were left for 48 hours for plaques to appear.

Plaques can be directly visualised by light microscopy but staining with cresyl violet acetate was used to enhance the plaques. To stain, the cells were fixed using 4% paraformaldehyde in PBS and then stained with 0.1% (w/v) cresyl violet acetate in 20% ethanol.

2.3.2 Infection of primary sensory neurons with the virus vector

The GFP containing virus was used in parallel, but in separate cells, with the HSP virus to gauge the level of infection. The multiplicity of infection (MOI) is the number of plaque forming units, infectious particles, used per cell. An MOI of 10 was used because the cells were sparsely plated and the virus adheres to the exposed plastic. The sensory neurons were infected in the same way as the MAM49 cell line. The virus was added to serum free media and then added to the cells and incubated at 37°C/ 95% humidity/ 5% CO_2 for 1hour. In the sensory neurons the media was then removed and

replaced with media containing serum. The cells were left for 24 hours to express the introduced gene. The viruses did not enter the lytic cycle in primary cells because the virus vector is disabled and the primary sensory neurons do not contain the complementing proteins.

2.4 Cardiotrophin-1

CT-1 was dissolved in water and added to the media at a concentration of 10ng/ml for 24 hours before cell stress was induced. Control cells were treated with the same volume of filter sterilized ddH_2O.

2.4.1 Chemical inhibitors used in conjunction with CT-1

A number of chemical inhibitors were used to block different signalling pathways in order to ascertain which pathways were activated by CT-1. The inhibitors were added to the media 1 hour before CT-1.

Inhibitor	Target	Final concentration	Diluent	Supplier
PD 98059	p42/44 MAPK	50μM	DMSO	NEB
SB 203580	p38 MAPK	10μM	DMSO	Sigma
LY 294002	PI-3 Kinase	10μM	EtOH	Sigma

Table 2.2:Chemical inhibitors used to block the effect of CT-1. PD 98059 inhibits the p42/44 MAPK pathway by inhibiting the activation of mitogen activated protein kinase kinase 1 (Cuenda et al., 1995; Alessi et al., 1995). SB 203580 inhibits the p38 MAPK pathway without affecting the p42/44 MAPK pathway (Cuenda et al., 1995). LY 294002 specifically inhibits PI-3 kinase without inhibiting PI-4 Kinase (Vlahos et al., 1994).

2.5 Urocortin

Urocortin was dissolved in 100% ethanol and then added to the media at a concentration of 0.5μg/ml ($1x10^{-7}$M) for 24 hours before the induction of cell stress. Control cells were treated with media containing the same volume of 100% ethanol.

2.6 Induction of cell stress

2.6.1 Heat shock

The normal heat shock response occurs 5°C or more above the normal physiological temperature. However, for heat shock to be lethal a greater stress is required. A variety of temperatures were tested in adult sensory neurons and 48°C for 10 minutes was found to be the most effective lethal heat shock. The plates were wrapped in parafilm and placed in a waterbath at 48°C for 10 mins, the cells were then returned to normal culturing conditions 37°C/ 95% humidity/ 5% CO_2.

2.6.2 Hypoxic ischaemia

The media was replaced with ischaemic buffer, modified from (Esumi et al., 1991), and placed in the hypoxic chamber. The hypoxic chamber is a cylinder made of Perspex with a heater and a thermostat to maintain 37°C. The lid has a rubber seal and is screwed down to ensure it is airtight. 5% CO_2 in argon is used to displace the oxygen (BOC gas). This method is designed to simulate the *in vivo* conditions during oxygen deprivation and has been described previously (Brar et al., 1999b).

Ischaemic buffer

137mM NaCl

12mM KCl

0.49mM $MgCl_2.4H_20$

0.9mM $CaCl_2.2H_20$

4mM HEPES

20mM Sodium lactate (DL lactic acid, sodium salt)

10mM deoxyglucose

H_2O to make up to 500mls

Adjust to pH 6.2 with 7.5% sodium bicarbonate (Invitrogen)

Control cells were maintained in the normal media in normal culturing conditions. After a period of hypoxic ischaemic stress in the hypoxic chamber the cells were returned to the normal incubator, 37°C/ 95% humidity/ 5% CO_2. Cell survival was measured 24 hours after the onset of stress, cells used for Tunnel were fixed after a 4 hour recovery period in the normal incubator.

2.7 Cell survival assay

Survival was measured using the morphology of the cells under phase contrast microscopy. The average number of cells that were alive after a treatment was compared to the average number that were alive before the treatment. The average was obtained by measuring 3 fields of view per well under x100 magnification for adult and aged cells and under x200 magnification for neonatal cells using an Axiovert 25, Zeiss microscope. To minimize observer bias the tissue culture plates were coded and scored "blind" in each experiment. Criteria for live and dead cells are listed in table 2.3 and have been previously described (Wyatt et al., 1996).

Criteria for live cells	Criteria for dead cells
Phase bright	Granular appearance
Smooth membrane	Rough or pitted membrane
Intact membrane	Broken membrane or blebbing
Neurites	Lack of neurites
Adherent	Not adherent

Table 2.3: Criteria used to determine whether cells were alive or dead.

2.8 Cell death assay

A modified TUNEL method adapted from (Gavrieli et al., 1992) was used to assess apoptotic cell death. In the TUNEL assay fluorescently labeled dNTPs are added to the 3'-OH groups of single or double stranded DNA fragments by Terminal transferase. The fluorescence was then visualized using normal fluorescent microscopy. As for the survival assay the tissue culture plates were coded and scored "blind" in each experiment to minimize observer bias.

The cells were fixed in 4% paraformaldehyde, after 20 minutes they were washed with PBS. The TUNEL reagents, Terminal transferase and tetramethyl-rhodamine-5-dUTP (Roche), were added to the cells. The reaction was incubated at 37°C for 1.5 hours in the dark. The TUNEL reagents were removed, the cells washed with PBS and the coverslips mounted onto microscope slides using DAKO (Insight Biotech) fluorescent anti-fade mounting medium. The slides were stored in the dark at 4°C until they could be examined.

To measure the amount of death, the cells were examined under phase contrast and under fluorescence using x200 magnification on a Axioskop 2 plus, Zeiss microscope. The two views were overlaid and the number of positively stained cells were counted and expressed as a percentage of the total number of cells for the same field of view. Three fields of view were measured for each well and the average taken.

For both the survival and death assays the data is expressed as the mean plus and minus the standard error of the mean, and compared using the Paired t-test and the Wilcoxon test.

2.9 Western Blot

2.9.1 Protein extraction

To extract protein, the cells were washed once in PBS to remove any media, cells were then scraped off the dish in cold PBS, and centrifuged at 13,000 rpm for 5mins at 4°C. The supernatant was discarded and the pellet resuspended in 0.1M Tris (pH8) with

0.5mM DTT and protease inhibitors (Roche). The samples were then subjected to 4 rounds of rapid freeze thawing in liquid nitrogen and a 37°C waterbath. At the end of each round the samples were vortexed. After all 4 rounds were completed the samples were centrifuged at 13,000 rpm for 10mins at 4°C. The pellet was discarded and the supernatant stored at -20°C.

2.9.2 SDS-PAGE

Proteins were separated into polypeptides using SDS polyacrylamide gel electrophoresis. The proteins are denatured and form negatively charged complexes with the detergent SDS in the gel. The electrical current causes the complexes to migrate through a highly porous stacking gel and then separate into separate polypeptides according to size in the resolving gel (Sambrook, 1989).

Stacking gel:

5% Acrylamide mix (30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide)
125mM Tris-HCl (pH6.8)
0.1% (w/v) SDS
0.1% (w/v) Ammonium persulphate
0.1% (v/v) TEMED

Resolving gel:

10% Acrylamide mix
375mM Tris-HCl (pH8.8)
0.1% SDS
0.05% Ammonium persulphate
0.05% TEMED

Tris-Glycine electrophoresis running buffer:

25mM Tris base

250mM Glycine (pH8.3)

0.1% SDS

Tris-glycine transfer buffer:

39mM Glycine

48mM Tris base

0.037% SDS

20% Methanol

2x SDS-PAGE loading buffer:

100mM Tris-HCl (pH6.8)

4% (w/v) SDS

0.2% (w/v) Bromophenol blue

20% (v/v) Glycerol

200mM Dithiothreitol (DTT)

The resolving gel was poured and then a layer of water saturated isobutanol added to the top to prevent oxygen diffusing into the gel. When the gel had set the isobutanol was poured off and the gel allowed to dry briefly before the stacking gel was poured. A comb was added before the gel polymerised.

Loading buffer was added to the protein samples that were then heated to 100°C for 5 minutes before loading into the gel. Rainbow protein markers (Amersham Bioscience) were used as well as Pre-stained broad range protein markers (NEB).

2.9.3 Equalisation of protein levels

Bradford assays were performed on the protein extracts to equalize the quantity of protein loaded into the SDS gels so that levels of proteins could be compared. In

addition each blot was reprobed with β actin to evaluate equal loading and transfer of the gels.

2.9.4 Transfer of the protein to membrane

The proteins were transferred from the gel to a Hybond-C membrane (Amersham biosciences) in tris-glycine transfer buffer using minigel transfer aparatus (Biorad) overnight at 150mA and 4°C.

2.9.5 Probing the membrane

A milk protein solution (5% Marvel and 1% Tween-20 in PBS) was used to block the membrane before probing with antibodies, to reduce non-specific binding of the antibodies.

The membranes were probed with antibodies in milk solution for 1 hour. After washes, in milk solution and PBS the membranes were probed with secondary antibody and washed again in milk solution and then PBS. The secondary antibodies were all conjugated to horse radish peroxidase (HRP) and came from DAKO, they were all used at 1 in 2000. The antibodies were detected using an ECL kit (Amersham Bioscience UK Ltd) and then visualised by exposing the membrane to photographic film (KODAK scientific imaging film) The film was developed by a Compact X4 (Xograph imaging systems). For a list of antibodies used see table 2.4 below.

2.9.6 Reprobing membranes

To reprobe the western blots the membranes were stripped in 1M Glycine (pH2.8) for 15 mins and then washed twice in PBS before blocking and reprobing.

Antibody	Secondary antibody	Size of protein detected	Source
Anti HSP 27 Goat polyclonal	HRP rabbit anti-goat	27KDa	Santa Cruz Biotechnology, inc.
Anti HSP 56 Goat polyclonal	HRP rabbit anti-goat	56KDa	Santa Cruz Biotechnology, inc.
Anti HSP 70 inducible Mouse monoclonal	HRP rabbit anti-mouse	70KDa	Stressgen Biotechnologies corp.
Anti HSP 90 mouse monoclonal (AC88)	HRP rabbit anti-mouse	90KDa	A kind gift from Dr D.A. Toft, The Mayo clinic, Rochester, USA.
Anti Actin Goat polyclonal	HRP rabbit anti-goat	43KDa	Santa Cruz Biotechnology, inc.
Anti Tubulin, neuronal class III, beta tubulin. Mouse monoclonal	FITC conjugated rabbit anti mouse	50KDa	Covance Research Products, Berkeley, California
Anti ERK 2 mouse monoclonal	HRP rabbit anti-mouse	42KDa	Santa Cruz Biotechnology, inc.
Anti phosphorylated ERK Mouse monoclonal	HRP rabbit anti-mouse	42KDa 44KDa	Santa Cruz Biotechnology, inc.

Table 2.4: Antibodies used for Westerns and Immunocytochemistry.

2.10 Immunocytochemistry

Antibody diluting buffer

5% Triton X-100 (V/V)

5% Foetal calf serum (V/V)

74mM NaCl

PBS

Cells were fixed in 4% paraformaldehyde and then pretreated with antibody diluting buffer to make the cells permeable. The neuronal tubulin antibody TUJ1 was used at 1:1000 in antibody diluting buffer overnight at 4°C. The secondary antibody was a FITC

conjugated anti mouse antibody and was used at 1:1000 in antibody diluting buffer for 1 hour at room temperature. The cells were cultured on coverslips and DAKO fluorescent anti-fade mounting medium was used to mount the coverslips onto microscope slides. The cells were photographed under phase contrast and under fluorescence using x200 magnification on a Axioskop 2 plus, Zeiss microscope.

2.11 Reverse transcription-Polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) allows the reverse transcription of mRNA to cDNA so that it can be amplified using the PCR, thus allowing the detection of actively transcribed genes in cells or tissues. Amplification from the cDNA can be distinguished from amplification of any contaminating genomic DNA by using primers that amplify a section of the gene including an intron, so the genomic copy will produce a much larger product.

2.11.1 RNA extraction

RNA extraction was carried out using Trizol (Invitrogen) and a standard Trizol protocol. Trizol was added to the cells or small pieces of tissue, mixed by pipetting, incubated for 5 minutes at room temperature and then transferred to an eppendorf. The small pieces of adult rat brain and liver were pulled through a 19 gauge needle in Trizol to break them up and maximize the amount of RNA extracted. After choloform was added, the tube was vortexed, incubated again at room temperature and then centrifuged. Isopropanol was then added to the aqueous layer and incubated on ice for 15 minutes. After centrifuging for 15 mins the pellet was washed with 70% ethanol, centrifuged again, dried and resuspended in RNase free water (Sigma).

2.11.2 Reverse transcription

Reverse transcription was carried out using AMV reverse transcriptase (Promega), dNTP and RNasin ribonuclease inhibitor (Promega) and 40mM sodium pyrophosphate

with 1µg of random hexamer primers and 5µl of RNA in a 25µl reaction as described by the AMV-RT protocol.

2.11.3 PCR

PCR was carried out using Promega Taq, dNTPs and $MgCl_2$. 0.5µl of cDNA was used for each 25µl reaction, 10mM dNTPs, 1.5mM $MgCl_2$ and 15pmols of each primer. 100pmols of each primer were used for the reaction to amplify CRHR2 along with betaine. The PCR reaction was run for 35 cycles with a 60°C 1 minute annealing step and a 72°C 30 second extension step. Specific primers were designed to CRHR1, CRHR2, gp130 and LIFR as well as peptidylprolyl isomerase A (Cyclophilin A) using the draft rat genome (ncbi) and Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) see table 2.5 below.

2.11.4 Visualization of the PCR product

The products of the PCR reactions were run on a 2% agarose gel along with a 100bp ladder and loading buffer (Promega). The DNA was then visualized by staining the gels in 0.5µg/ml ethidium bromide in TAE for 15 – 30 minutes and then destaining the gel in water for 10 – 20 mins. The gels were photographed under UV light.

2.11.5 Controls

Adult rat brain was used as a positive control for CRHR1 and CRHR2 as both receptors are known to be expressed in the brain (Chang et al., 1993; Perrin et al., 1993; Lovenberg et al., 1995b). Adult rat liver was used as the positive control for gp130 and LIFR as they were both originally isolated from liver and liver is the organ of choice for extracting RNA (Wang et al., 1992; Tomida et al., 1993; Aikawa et al., 1997).

All the RT-PCR reactions were run in parallel with and without the reverse transcriptase, reactions run without reverse transcriptase were used as negative controls to ensure the PCR product was not amplified from any contaminating genomic DNA. The primers were also intron spanning so that any amplification from the genomic DNA

could be identified by the size of the product. For each set of primers a PCR reaction with just water and no template was run to ensure none of the other reagents were contaminated with DNA of any kind.

Gene amplified	Primers used	Expected product	Position in gene	Genbank acession number
gp130	Forward: 5' ACC ATGTTG CCG TTC CTA AG 3' Reverse: 5' GTC AAC ATC GAG GTC TGG GT 3'	382bp	Exons 2 - 4	A44257
LIFR	Forward: 5' GCT GAC TTC TCG ACC TCC AC 3' Reverse: 5' GGT ATT ACG CGT CCA GGA AA 3'	315bp	Exon 4 – 5/6	D86345
CRHR1	Forward: 5' GTG GAT GTT CGT CTG CAT TG 3' Reverse: 5' CGG AGT TTG GTC ATG AGG AT 3'	207bp	Exons 5 - 7	NP_112261
CRHR2	Forward: 5' TTT TCC TAG TGC TGC GGA GT 3' Reverse: 5' AGC CTT CCA CAA ACA TCC AG 3'	217	Exons 4/5 - 6	NP_073205
Cyclophillin A	Forward: 5' AGC ACT GGG GAG AAA GGA TT 3' Reverse: 5' AGC CAC TCA GTC TTG GCA GT 3'	243bp	Exons 3 - 4	NM_017101

Table 2.5: Genes amplified in PCR reactions and primers used.

Chapter 3: The protective effect of Cardiotrophin-1 in neonatal and adult sensory neurons.

3.1 Introduction

Cardiotrophin-1 (CT-1), a cytokine in the IL-6 family, was originally identified in the heart as an inducer of cardiac myocyte hypertrophy (Pennica et al., 1995a). It was later shown that CT-1 caused hypertrophy through the receptor components glycoprotein 130/Leukemia inhibitory factor receptor (gp130/LIFR) (Wollert et al., 1996). CT-1 protects cardiac myocytes *in vitro* against the cell death caused by ischaemia reperfusion (Stephanou et al., 1998a; Brar et al., 2001b). CT-1 also protects against ischaemic damage in whole hearts *ex vivo* (Liao et al., 2002). Several signalling pathways appear to be involved in the effect of CT-1 in the heart, p42/44 MAPK, p38 MAPK, PI3-Kinase and NF-kappa B have all been shown to be activated by CT-1 treatments (Sheng et al., 1997; Kuwahara et al., 2000; Craig et al., 2001; Brar et al., 2001a; Liao et al., 2002).

Heat shock proteins are important to protect cells against the potentially lethal damage caused by stresses such as periods of ischaemia. CT-1 treatment increases heat shock protein expression, although there is some disagreement in the literature about the level of expression achieved. This may be due to differences in the level of heat shock proteins in the control cells since experimental manipulation can induce heat shock protein expression and this may differ between experiments depending on the amount of stress the cells are exposed to during preparation and during experiments. CT-1 treatment resulted in a three fold induction of HSP 70 and a seven fold induction of HSP 90 in neonatal cardiac myocytes in culture (Stephanou et al., 1998a). However Railson et al (2000) reported a 4 fold increase in HSP 90 expression after CT-1 treatment in CLEM cells (a cardiac myocyte cell line) and a 2.9 fold increase in HSP 90 after CT-1 treatment in cultured neonatal cardiac myocytes. The increase in heat shock protein 90 levels reported by Railson et al (2000) occured at the post transcriptional level as it did not require de novo transcription (Railson et al., 2000). No increase in HSP 70 levels

was observed after CT-1 treatment either in the CLEM cells or the cardiac myocytes despite the higher concentration of CT-1 used in these experiments, 2ng/ml as opposed to 1ng/ml used by Stephanou et al (1998).

Heat shock induces high levels of heat shock protein expression but CT-1 treatment and heat shock together result in a higher level of heat shock protein 90, a 6 fold increase, in CLEM cells compared to CT-1 treatment alone, which inducs a 4 fold increase, but a lower level than heat shock alone, which induces a 20 fold increase in heat shock protein 90. In cultured neonatal cardiac myocytes this effect is much smaller, CT-1 treatment alone inducs a 2.9 fold increase in HSP 90 levels whilst heat shock inducs a 4 fold increase and the two treatments together cause a 3 fold increase in HSP 90 levels (Railson et al., 2000). The induction of heat shock protein expression by heat shock and by the combination of CT-1 treatment and heat shock is dependent on transcription in both CLEM cells and cultured neonatal cardiac myocytes because it is prevented by the RNA synthesis inhibitor actinomycin D (Railson et al., 2000). This is very interesting because CT-1 treatment appears to reduce the level of stress experienced by the cells during heat shock, this may be because the presence of higher levels of heat shock proteins before the cells are exposed to stress allows more efficient protection by the heat shock proteins. Perhaps maximal induction of the heat shock proteins by the cells is not achieved during heat shock after CT-1 treatment because it is not necessary. Therefore it was proposed that CT-1 could be a useful non-stressful way to increase the levels of heat shock proteins and therefore protect cells against stress. This would be particularly useful in older animals because the normal heat shock protein response becomes impaired with age and this could be a way to bypass that impairment (Fawcett et al., 1994).

As well as its role in the heart, CT-1 is an important physiological factor in motor neurons. Pennica et al (1996a) propose CT-1 as a physiological target derived neurotrophic factor during development for motor neurons; it can be synthesized and secreted by immortalized embryonic muscle, the LIF receptor is expressed in the limb bud and CT-1 mRNA was found at high levels in the limb bud at E13.5 and it decreases during the normal period of motor neuron death.

Knockout mice have been produced to investigate the role of the IL-6 family members CT-1, Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) in neurons. Mice have been produced lacking each of the three proteins as well as a combined knockout of LIF and CNTF and knockouts of the receptor components gp130, LIFR and CNTFRα.

IL-6 knockout mice show a decrease in the relative size distribution of the sensory neurons and a reduction in temperature sensitivity, as well as delayed functional recovery after a crush injury and loss of recovery of the motor neurons after a crush injury (Zhong et al., 1999). IL-6 expression increases in sensory neurons after nerve injury (Murphy et al., 1995; Kurek et al., 1996; Reichert et al., 1996). Surprisingly, IL-6 does not protect sensory neurons in vitro unless the IL-6-α receptor is also delivered to the cells (Murphy et al., 1995; Simon et al., 1995; Kurek et al., 1996; Thier et al., 1999b).

In CT-1 knockout mice there is increased death of the motor neurons between E14 and P9, but there is no further loss of motor neurons compared to control animals after P9 and lesions in young adults do not result in further loss of motor neurons (Oppenheim et al., 2001).

Deletion of CNTF or LIF does not result in loss of motor neurons during development. However there is some postnatal degeneration of the motor neurons in CNTF mouse knockouts (Masu et al., 1993; Sendtner et al., 1996). The LIF knockout mouse reveals no noticeable pathology except suppression of the injury induced expression of the neuropeptides vasoactive intestinal peptide (VIP), neurokinin A and Galanin in the superior cervical ganglia. The injury response is not restored by treatment with exogenous LIF suggesting that either the exogenous LIF is not able to act on the cells in the same way that endogenous LIF can or that the lack of LIF during development causes a change in the cells (Rao et al., 1993). The loss of injury induced expression of these neuropeptides in LIF knockout mice suggests that LIF is involved in a common pathway involving all three peptides and that this pathway is an important part of the neuronal response to injury. The LIF CNTF double knockout has more severe motor neuron degeneration than the CNTF single knockout suggesting that endogenous LIF is

able to compensate, at least partially, for the lack of CNTF (Sendtner et al., 1996). However in progressive motor neuronopathy mice (pmn) nerve lesion and the subsequent expression of CNTF prevents the expected death of motorneurons, but in pmn CNTF -/- mice the predicted upregulation of LIF does not occur and therefore does not compensate for the lack of CNTF (Sendtner et al., 1997).

Exogenous LIF is able to prevent neonatal sensory neurons becoming apoptotic following axotomy *in vivo*. However, the protective effect of LIF only lasts for 7 days suggesting that rather than preventing the death of the cells the LIF treatment delays it. Perhaps further treatments with LIF would keep the cells alive for longer. However if they have entered a pre-apoptotic state they may have survived but not have been fully functional (Cheema et al., 1994).

Although CT-1, LIF and CNTF appear to be important, mice lacking the receptors have much greater pathology. Signalling by CT-1, LIF and CNTF is mediated by heterodimerization of gp130 and LIFR plus an alpha component for CT-1 and CNTF called CT-1Rα and CNTFRα respectively. Loss of both sensory neurons and motor neurons is observed in knockout mice lacking the receptor component gp130 (Nakashima et al., 1999). In marked contrast to the LIF and CNTF knockouts where relatively little pathology was seen, absence of either of the receptor components (LIFR and CNTFRα) is perinatally fatal (Masu et al., 1993; DeChiara et al., 1995; Li et al., 1995; Ware et al., 1995). The LIFR knockout shows a significant loss of motorneurons and astrocytes (Li et al., 1995; Ware et al., 1995). The CNTFRα knockout also has severe motor neuron deficits (DeChiara et al., 1995). It is clear that there is some redundancy in the IL-6 family of ligands but that signalling through the receptors is crucial both for the development of sensory and motor neurons and for the maintenance of sensory neurons.

As in the heart, CT-1 has a protective effect in motor neurons both *in vitro* and *in vivo*. In mice CT-1 gives protection against cell death following axotomy *in vivo* as well as supporting the long term survival of a large subset of motor neurons *in vitro* (Pennica et al., 1996a). Neurons *in vitro* have been axotomized in order to culture them which is stressful. CT-1 also reduces the pathological degeneration of neurons observed in some

diseases. In progressive motor neuronopathy (pmn) mice CT-1 significantly reduces the anticipated pathological degeneration when delivered in an adenoviral vector and when delivered by electroporation (Bordet et al., 1999; Lesbordes et al., 2002). CT-1 also has a protective effect in a mouse model of amyotrophic lateral sclerosis (ALS) when delivered via an adenoviral vector (Bordet et al., 2001b). CT-1 also confers protection in a model of spinal muscular atrophy where part of the survival motor neuron gene SMN1 has been deleted (Lesbordes et al., 2003).

CT-1 has a trophic effect in sensory neurons, as in motor neurons, promoting survival of neonatal rat dorsal root ganglia sensory neurons in culture (Thier et al., 1999a). CT-1 treatment at a concentration of 100ng/ml results in a 5 fold increase in the number of cells surviving in culture after 50 hours, compared to cells kept in serum free media without NGF. NGF treatment results in a 5 fold increase in the number of cells surviving at 10ng/ml compared to the control cells maintained in serum free media without exogenous trophic factors. Thier et al (1999a) report that approximately 10% of the control cells originally seeded survived to 50 hours. CT-1 also promotes survival of dopaminergic neurons and ciliary neurons (Pennica et al., 1995b).

CT-1 has a protective effect in cardiac myocytes and leads to increased levels of the heat shock proteins HSP 90 and HSP 70 (Stephanou et al., 1998a). CT-1 delivered by adenovirus vector to PC12 cells protects against excitatory damage, oxidative stress and apoptosis via the same signalling pathways, PI-3 Kinase and MAPK, that are activated by CT-1 in the heart (Toth et al., 2002). In motor neurons it has a trophic effect and protects the cells against the stress of physical damage and pathological degeneration. In sensory neurons it also has also a trophic effect but beyond this the effect of CT-1 on sensory neurons has not been examined (Thier et al., 1999a). It was proposed that CT-1 would have a much greater effect on sensory neurons than the trophic effect described. The aims of this chapter were to investigate whether CT-1 could protect sensory neurons against stressful conditions such as hypoxic ischaemia, and to investigate whether it could induce heat shock proteins in sensory neurons. The ability of CT-1 to induce heat shock protein expression in motor neurons has not been tested. If CT-1 induced heat shock protein expression in sensory neurons then it could potentially

provide a way to overcome the age associated impairment of the induction of heat shock proteins by increasing the levels of HSPs in a non-stresful way in neurons.

3.2 Methods

In order to investigate whether CT-1 could protect sensory neurons against stress, the previously demonstrated trophic effect had to be controlled for, so that any differences in survival would not be due to greater death in the untreated cells rather than greater survival in the CT-1 treated cells. To achieve this, the number of cells alive after 48 hours in culture was measured and this measurement was used as a baseline for comparison with the number of cells surviving 24 hours after the stress. This also controlled for any other factors affecting survival before the crucial 24 hour period, see figure 3.1 for an outline of the experimental protocol used. Neonatal sensory neurons were cultured with NGF in the media because they are dependent on NGF and a large subset of DRG sensory neurons do not survive without it. It would not be feasible to study the effect of potentially protective agents on these cells if only 10% of the control cells survived the culturing process. Adult cultures were cultured without specific additional trophic factors, but in the presence of foetal calf serum, because they are not dependent on NGF for survival (Lindsay, 1988). Ischaemia was chosen as the stress because it is more physiological than chemical stresses and CT-1 has been shown to protect against it in cardiac myocytes (Stephanou et al., 1998a).

The cells were cultured and maintained at 37°C/ 95% humidity/ 5% CO_2 as described in the materials and methods. After 24 hours in culture the cells were treated with CT-1, the CT-1 was left in the media for 24 hours. The number of cells alive was measured, the cells were stressed and left to recover. After a further 24 hours the number of cells alive was measured again.

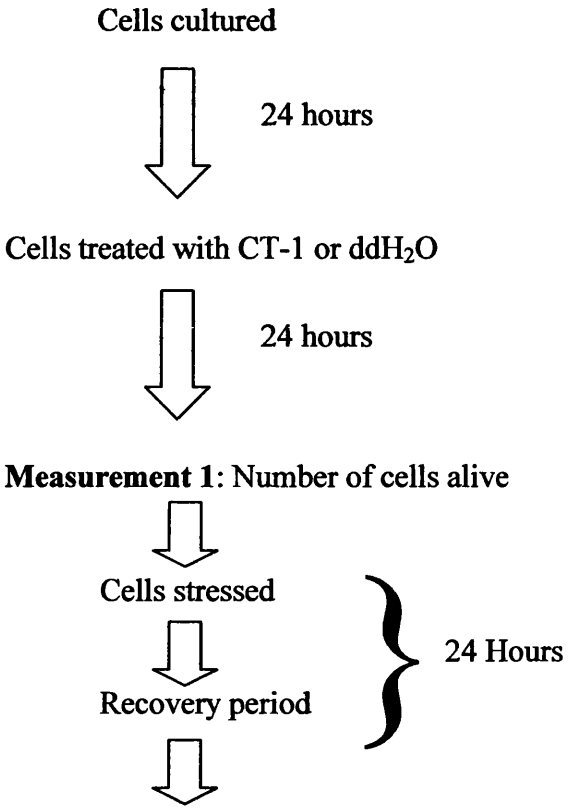


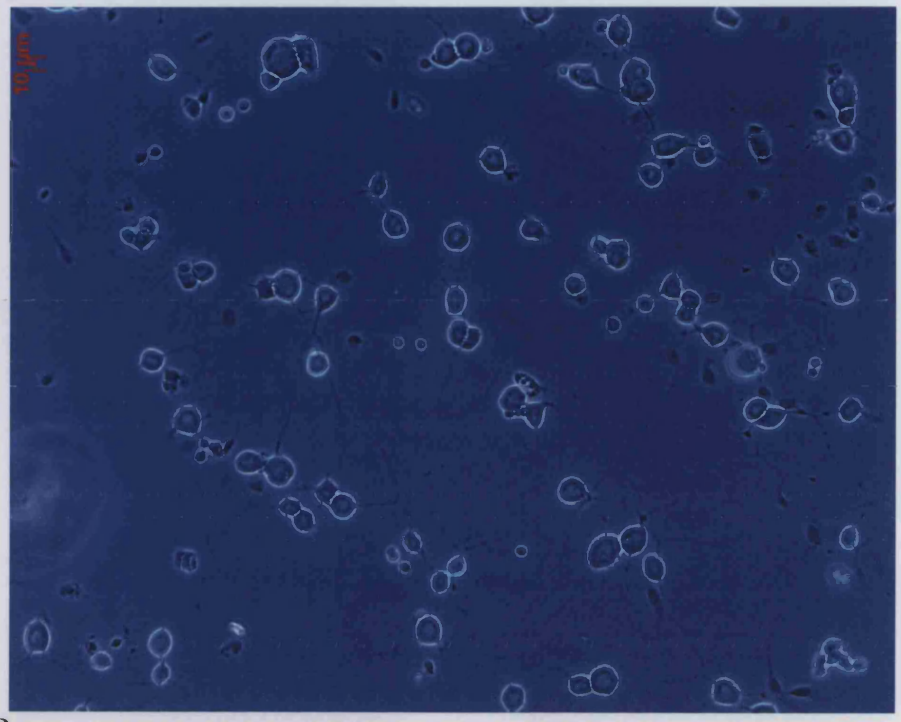
Figure 3.1: Experimental protocol for measuring cell survival. Illustrating the time frame for the different steps in the experiment and when measurements were taken.

The percentage survival was obtained by measurement 2/measurement 1 multiplied by 100. By using this method any factors affecting the survival of the cells outside the crucial 24 hour period between the two measurements were controlled for.
CT-1 was used at a final concentration of 10ng/ml. In the experiments done in the heart CT-1 was used at 1ng/ml and 2ng/ml, see 3.1. Thier et al (1999a) showed that CT-1 was more protective than CNTF at 10ng/ml and more protective than NGF at 100ng/ml resulting in a 4.5 and 5 fold increase in survival respectively. There was no increase in the level of survival when 1μg/ml was used (Thier et al., 1999a). Hence, 10ng/ml was used as the final concentration in experiments discussed in this chapter because it was within the concentrations previously shown to be effective and produced an almost maximal effect in sensory neurons (Thier et al., 1999a).

3.3 Hypoxic ischaemia in sensory neurons

Cultures of neonatal sensory neurons were set up as described in the materials and methods and they exhibited normal neuronal morphology, i.e a round phase bright cell body with neurites. In addition, immunocytochemistry was carried out using an antibody to neuronal class III β-Tubulin (TUJ1) to confirm that the cells were neuronal. The TUJ1 antibody is specific for neuronal tubulin and does not detect glial tubulin (Lee et al., 1990), this confirmed that the cells were neuronal. Figure 3.2 shows the results of the staining for a sample field of view.

A



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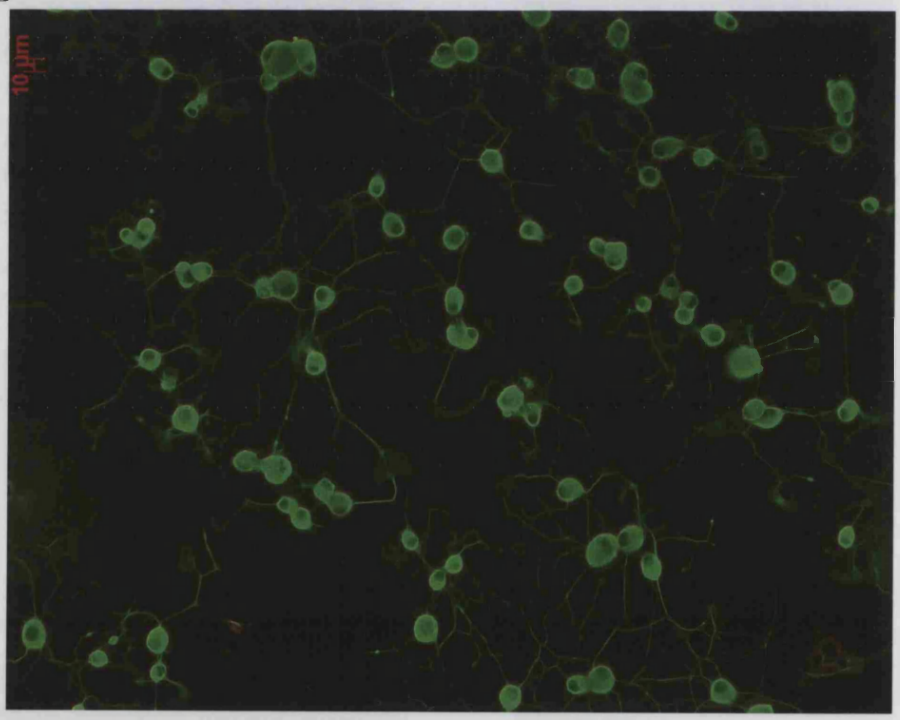

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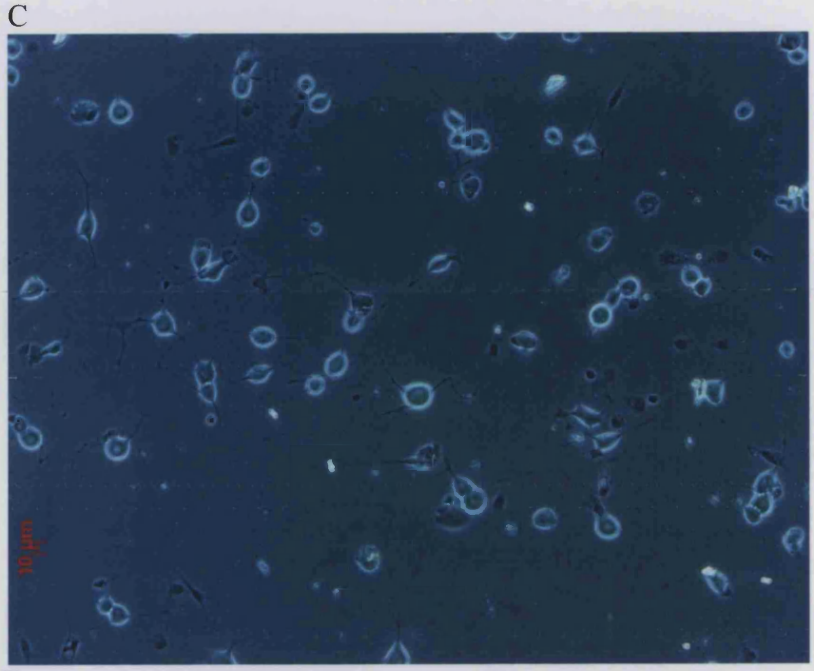

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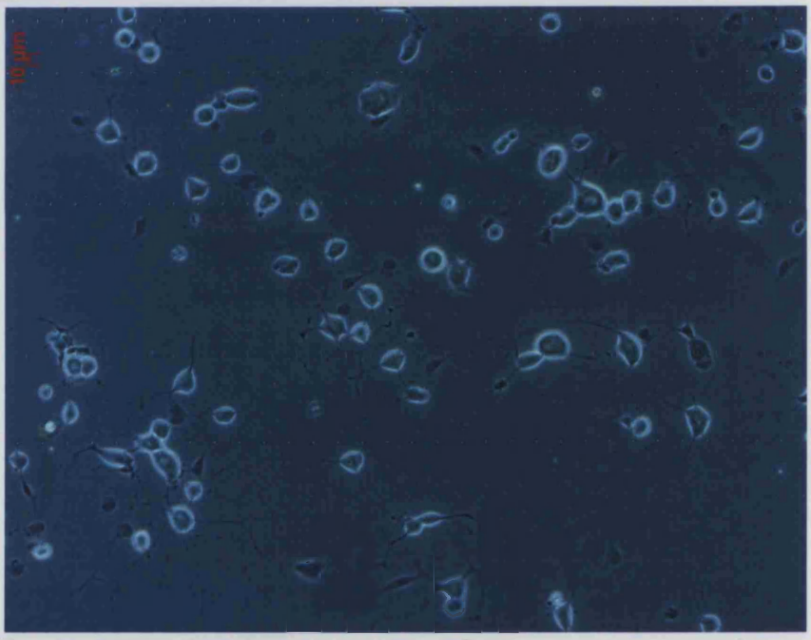


Figure 3.2: Immunocytochemistry on neonatal sensory neurons using a neuronal marker. Staining with a neuronal class III β-Tubulin monoclonal antibody and a FITC secondary antibody was used to visualize the tubulin antibody. The staining shown above is A) the cells under phase contrast and B) the same field of view with the antibody staining, C) and D) show cells under phase contrast overlaid with the fluorescent field of view for staining done with the primary antibody only and the secondary antibody only respectively. The primary antibody was used at 1:1000, magnification x200.

Having established that the cells were sensory neurons, a time course of simulated ischaemia was carried out for both the neonatal and the adult sensory neurons in order to establish the conditions needed to test the protective effect of CT-1. Figure 3.3 shows the survival of neonatal sensory neurons after a variety of periods of ischaemic stress and 24 hours recovery, figure 3.4 shows the survival of adult sensory neurons. Although the time courses suggested that a 3 hour stress would be the most suitable, in subsequent experiments 4 hours of stress was found to be necessary, perhaps the manipulation of the cells for experiments caused a degree of stress and therefore some preconditioning.

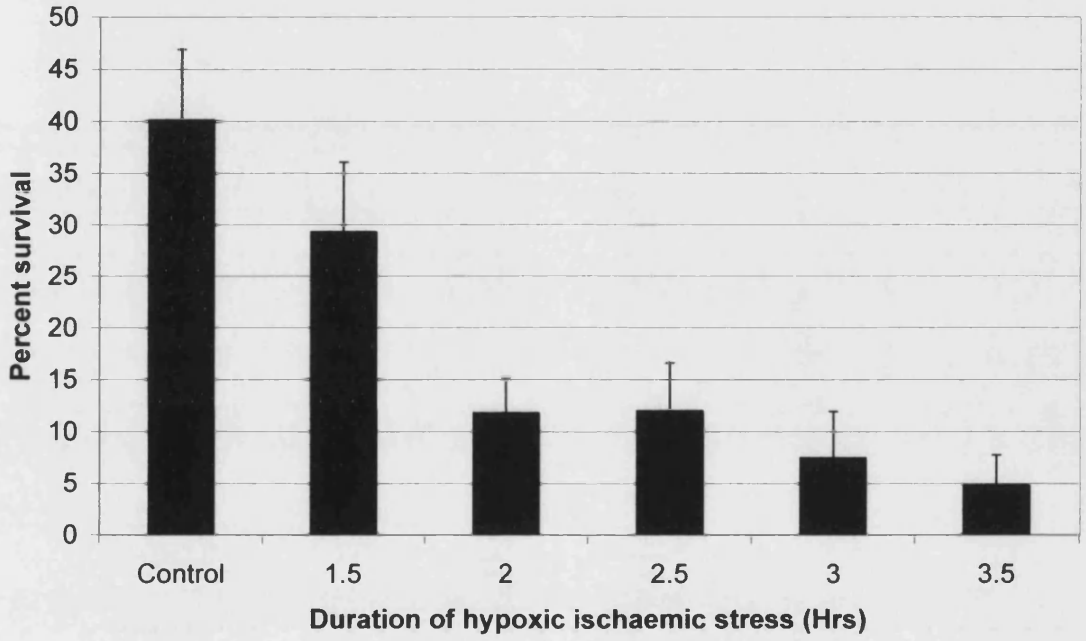


Figure 3.3: Time course of hypoxic ischaemic stress in neonatal sensory neurons. Cells were subjected to a hypoxic ischaemic stress for the indicated time. The ischaemic buffer was replaced with normal growth media and the cells were returned to 37°C, 95% humidity and 5% CO_2 for 24 hours before survival was measured. Survival was measured by morphology as described in the materials and methods, n = 1. The error bars show the standard deviation within one experiment, each time point was repeated 6 times.

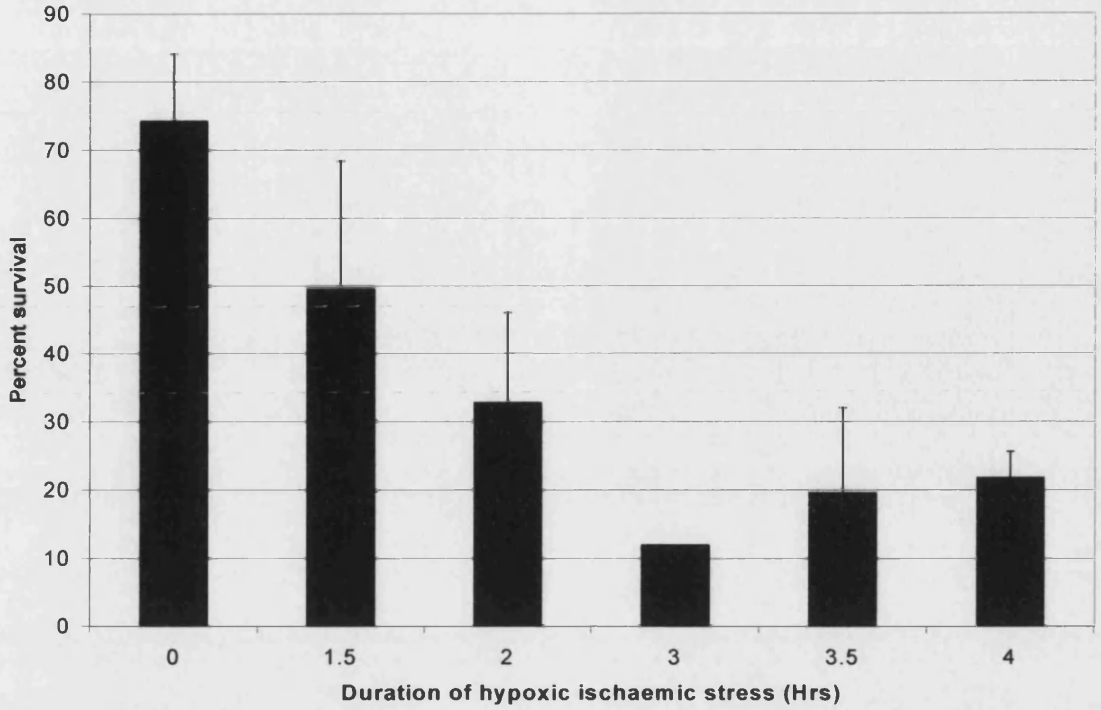


Figure 3.4: Time course for hypoxic ischaemic stress in adult sensory neurons. Cells were subjected to a hypoxic ischaemia stress for the indicated time, the ischaemic buffer was replaced with normal growth media and the cells were returned to 37°C, 95% humidity and 5% CO_2 for 24 hours before survival was measured. Survival was measured by morphology, n = 2 for all time points except 0 where n = 5 and 3 hours where n = 1, error bars are shown where appropriate and represent the standard error of the mean.

3.4 The effect of CT-1 in neonatal sensory neurons

Having established the conditions necessary, the ability of CT-1 to protect neonatal sensory neurons against a lethal ischaemic stress was tested. CT-1 was added to the cells 24 hours before they were subjected to the stress and survival was measured by morphology 24 hours after the stress as described in 3.2. The morphology was examined after 24 hours because the morphological changes occur late in cell death.

Figure 3.5 shows that CT-1 significantly increased survival of the neonatal sensory neurons subjected to 4 hours of hypoxic ischaemia ($p=0.03$). CT-1 protected approximately 10% of the sensory neurons against a lethal stress. This was not due to

there being more cells alive at the beginning of the experiment because the survival after stress was a percentage of the number of cells alive immediately before the cells were exposed to the stress. There was no increase in survival in the CT-1 treated control cells indicating that the increase in survival in the stressed cells was not due to the general trophic effect of CT-1, otherwise you would expect to see an increase in the survival of the control cells as well.

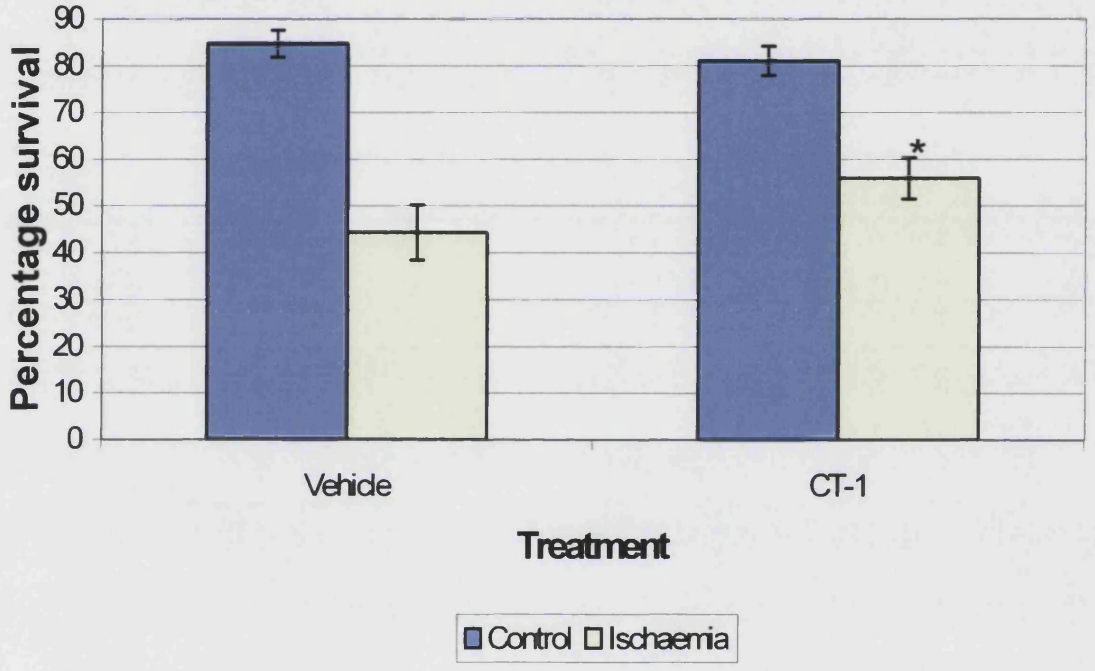


Figure 3.5: CT-1 protects neonatal sensory neurons against a hypoxic ischaemic stress. As described in the materials and methods the cells were cultured in DMEM with 10% foetal calf serum and survival was measured by morphology, n = 11, * p = 0.03 CT-1 after ischaemia versus vehicle after ischaemia. The error bars represent the standard error of the mean.

The experiment was repeated using the TUNEL assay to measure the number of cells undergoing apoptosis. For the TUNEL assay, the cells were fixed in 4% paraformaldehyde 4 hours after the stress. This was sufficient time for cell death to have begun and DNA fragmentation to have occurred (Gavrieli et al., 1992).

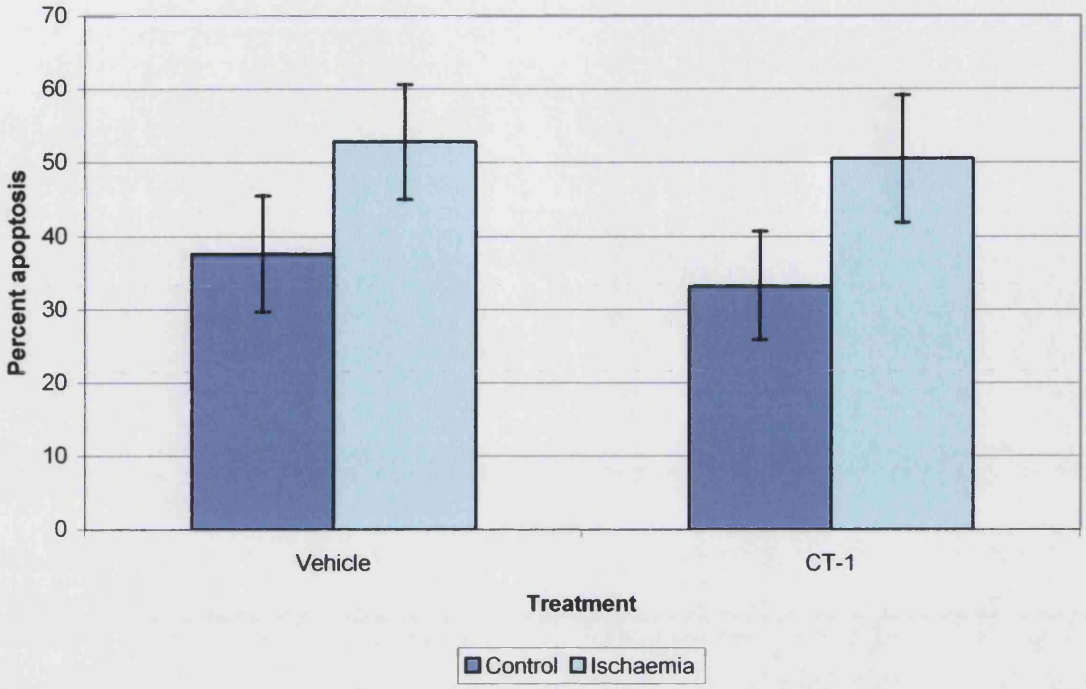


Figure 3.6: The protective effect of CT-1 in neonatal sensory neurons as measured by TUNEL. The TUNEL assay was performed as described in the materials and methods. The cells were cultured in DMEM supplemented with 10% foetal calf serum, n = 9, the error bars represent the standard error of the mean.

Figure 3.6 shows that CT-1 did reduce the level of DNA fragmentation after a lethal ischaemic stress, suggesting that CT-1 was reducing the amount of apoptotic death, but the reduction was not significant. Since the level of apoptosis recorded was equal to the amount of death seen in the survival assay of the untreated cells (55% death in the survival assay and approximately 55% apoptosis) and slightly greater than the death observed in the survival assay of the CT-1 treated cells (45% death in the survival assay and 50% apoptosis) the ischaemic stress was resulting in apoptosis. In the control cells not exposed to ischaemia there was approximately 35% apoptosis compared to only approximately 15% death in the controls of the survival assay. The morphological changes during cell death occur after, and as a result of, the molecular and biochemical changes, therefore the cells which stained positive in the TUNEL assay would appear dead later in the survival assay. However the TUNEL assay was performed after a 4 hour recovery period whereas the survival was measured after a 24 hour recovery period

to allow the morphological changes, which are later events than the DNA fragmentation, to occur.

As predicted there was less death in the control cells treated with CT-1 than in the untreated control cells, this was because the TUNEL assay detects all the death that has occurred since the cells were cultured, a baseline for the death was not taken before the cells were exposed to stress because the cells have to be fixed for the TUNEL assay and the assay would affect the survival of the cells if it was done on unfixed cells. For the TUNEL assay the percent death was obtained by dividing the number of positively stained cells by the total number of cells present in the field of view and multiplying by 100. Therefore you would expect to see not only the protective effect of CT-1 observed in the survival assay but the trophic effect of CT-1 previously reported as well.

The conditions used to maintain cells *in vitro* can affect the behaviour of the cells. To investigate whether the effect observed with CT-1 was dependent on the culturing conditions, cells were cultured in F14 media and the effect of CT-1 was tested again. F14 is defined media and is supplemented specifically with all the factors the cells need rather than supplementing with serum where the detailed contents are not known. Importantly, as shown in figure 3.7, the culturing conditions did not affect the protection by CT-1 seen in neonatal sensory neurons.

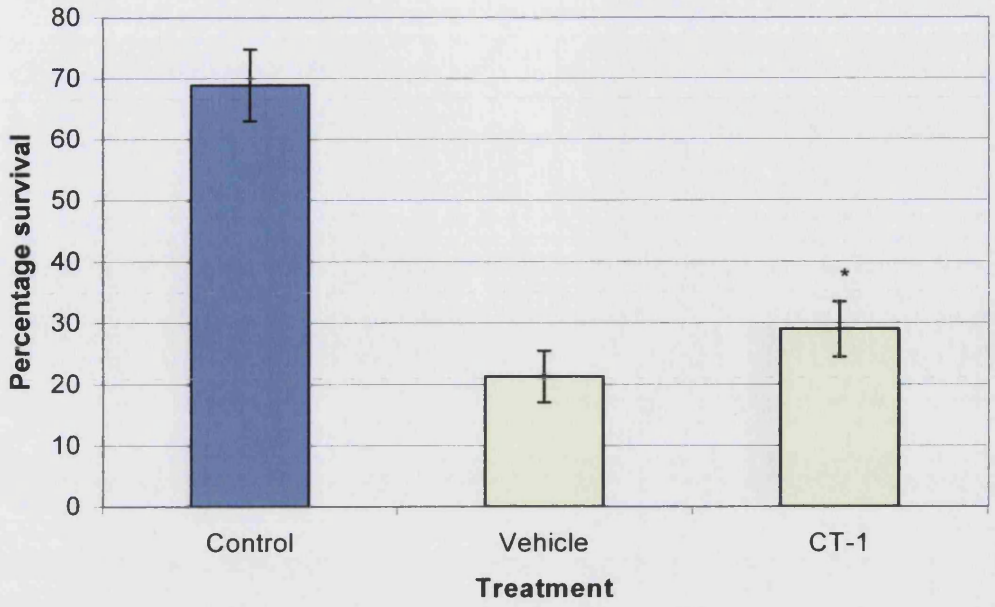


Figure 3.7: Culturing conditions do not affect the protective effect of CT-1 in neonatal sensory neurons. Cells were cultured in F14 without serum as described in the materials and methods and subjected to a 4 hour hypoxic ischaemic stress as for figures 3.5 and 3.6. Survival was measured by morphology, the blue bar represents survival of the control cells and the yellow bars represent the survival after ischaemia n = 7, * p = 0.02 CT-1 after ischaemia versus vehicle only after ischaemia. The error bars represent the standard error of the mean.

3.5 The effect of CT-1 in adult sensory neurons

Although CT-1 had a protective effect in neonatal sensory neurons no such effect was observed in adult cells as shown by figure 3.8 where the cells were cultured in F14 supplemented with 10% foetal calf serum as described in the materials and methods. As for the neonatal cells the hypoxic ischaemic stress was for 4 hours.

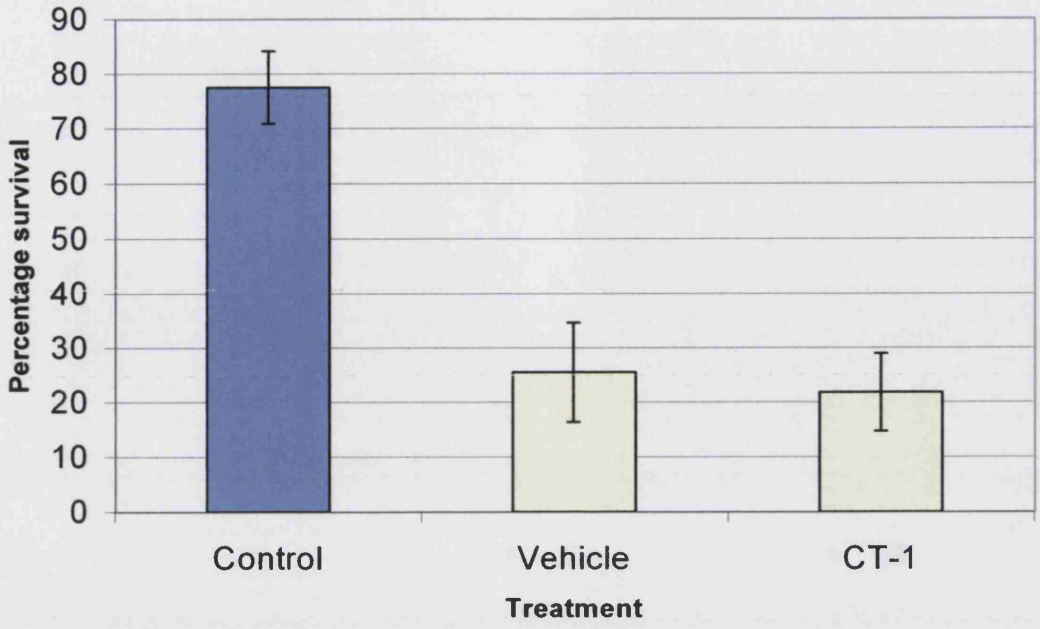


Figure 3.8: CT-1 does not protect adult sensory neurons against a hypoxic ischaemic stress. The cells were cultured in F14 media with serum, the blue bar represents survival of the control cells and the yellow bars represent the survival after ischaemia, n = 5, the error bars represent the standard error of the mean.

3.6 The mechanism of protection

Since CT-1 had a protective effect in neonatal cells but not in adult cells, RT-PCR was performed to determine the presence of the mRNA for two of the receptor components of CT-1 in both age groups, figure 3.9. The sequence for the proposed third component, CT-1Rα, is unknown. The cells were cultured as normal and left for 24 hours in the normal culturing conditions, in DMEM media, before RNA extraction. Adult Liver tissue was used as a positive control because both gp130 and LIFR were originally cloned from the liver (Wang et al., 1992; Tomida et al., 1993).

Figure 3.9 shows that the mRNA for the receptor components gp130 and LIFR was present in both the neonatal and adult cells. The cyclophilin A PCR reactions show that there was cDNA present for all three RT reactions, adult liver, adult sensory neurons and neonatal sensory neurons. All the negative controls were blank revealing that there was no contamination in any of the reactions. Only single bands of the predicted sizes (See table 2.5) were visible on the gel and the primers were designed so that any amplification product from genomic DNA would be much larger, revealing that there was no amplification from genomic DNA in any of the reactions. These were not quantitative PCR reactions so no conclusions can be drawn about the levels of mRNA in the cells. The only conclusion that can be drawn is that there was mRNA present for both gp130 and LIFR in both adult and neonatal sensory neurons. This suggests that there may be protein present in the cells and therefore a functional CT-1 receptor. As no differences were found between the presence of mRNA in the neonatal cells and in the adult cells this does not explain the difference between the protective effect of CT-1 in the neonatal cells and the lack of a protective effect in the adult cells.

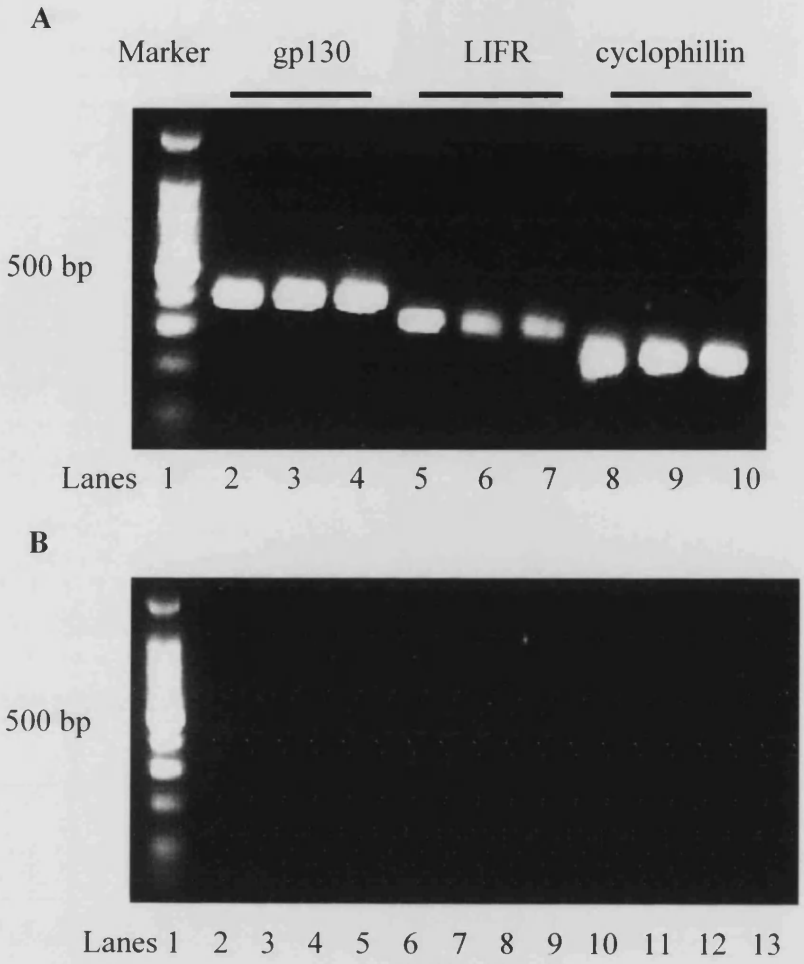
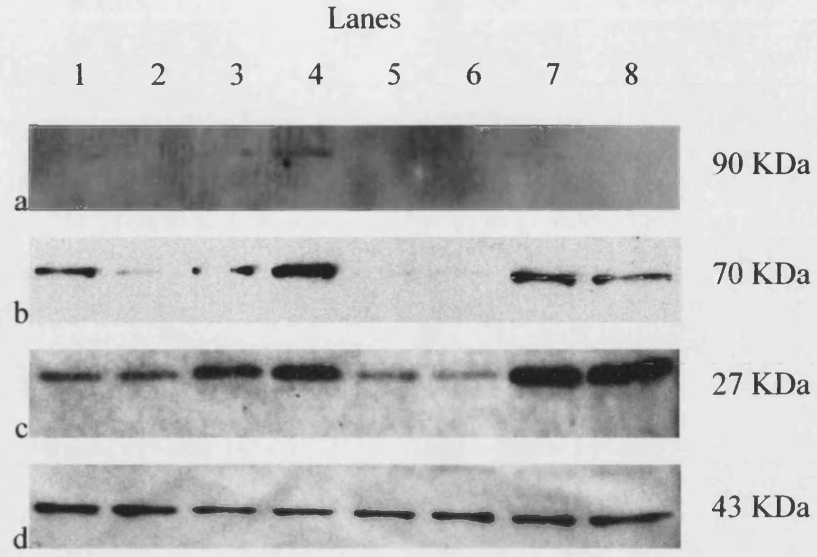

A
Marker
gp130
LIFR
cyclophillin
500 bp
Lanes 1 2 3 4 5 6 7 8 9 10
B
500 bp
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 3.9A mRNA for the receptor components gp130 and LIFR are expressed in adult and neonatal sensory neurons, results for the test samples. Lane 1 contained the promega 100bp ladder, the brighter band is 500bp and is more intense to allow orientation of the markers. Lane 2 contained the reaction product for gp130 from adult liver, this was used as a positive control for both the gp130 and the LIFR reactions. Lane 3 contained the reaction product for gp130 from adult sensory neurons and lane 4 the gp130 product from neonatal sensory neurons. Lane 5 contained the reaction product from adult liver for LIFR. Lanes 6 and 7 show the reaction product for LIFR from adult and neonatal sensory neurons respectively. Lanes 8, 9 and 10 show the reaction product for cyclophilin A from adult liver, adult sensory neurons and neonatal sensory neurons respectively.

Figure 3.9B mRNA for the receptor components gp130 and LIFR are expressed in adult and neonatal sensory neurons, results for the control samples. Lane 1 contained the same promega 100bp ladder as in 3.6.1A. Lanes 2 – 4 contained the PCR products for reactions run with the gp 130 primers and adult liver, adult sensory neurons and neonatal sensory neurons minus RT reaction products. Lane 5 contained a no template, water only reaction for the gp 130 primers. Lanes 6, 7 and 8 contained the PCR products from minus RT reactions for LIFR from adult liver, adult sensory neurons and neonatal sensory neurons respectively. Lane 9 was the no template, water only, reaction for LIFR. Lanes 10, 11 and 12 were the minus RT reactions for cyclophillin A from adult liver, adult sensory neurons and neonatal sensory neurons respectively and lane 13 was the no template control for cyclophillin A. 5µl of a 25µl reaction were run for all reactions.

Western blotting was carried out to establish whether CT-1 was inducing endogenous heat shock protein expression as it has been shown to in cardiac myocytes (Stephanou et al., 1998a). If CT-1 was increasing heat shock protein levels, this could suggest a potential protective mechanism and perhaps explain the differences between the effect of CT-1 in the neonatal cells and in the adult cells.

Figure 3.10 shows that CT-1 did not increase heat shock protein levels in neonatal sensory neurons. HSP 90 and HSP 70 levels were higher in adult cells treated with CT-1 for 1 hour (Lane 4a and b) but had decreased again by 24 hours (Lane 8a and b). There was no increase in the levels of HSP 90 or HSP 70 in neonatal cells treated for 1 hour or 24 hours (Lanes 2 a and b and 6 a and b respectively). There was no increase in the level of HSP 27 in the neonatal or adult cells in response to CT-1 (c) and the level of HSP 56 remained below the level of detection for all treatments (Not shown). Hence, although heat shock protein levels were increased in the adult cells this was not protective. The β-actin band shows approximately equal loading of the proteins and transfer to the membrane.



Treatment:	water CT-1	water CT-1	water CT-1	water CT-1
Age:	Neonatal	Adult	Neonatal	Adult
Time:	1 hour	1 hour	24 hours	24 hours

Figure 3.10 HSP protein expression in adult and neonatal sensory neurons treated with CT-1. The same western blot was reprobed with; a) HSP 90, b) HSP 70, c) HSP 27 and d) β-actin antibodies. HSP 56 was also probed but levels were undetectable in all samples (Not shown). The markers used were broad range, pre-stained NEB markers and the approximate MW of the bands is shown on the right side of the western blots and was determined from the molecular weight markers. Lane 1 contained protein extracted from neonatal sensory neurons after a 1 hour treatment with water alone, lane 2 contained proteins extracted from neonatal sensory neurons after a 1 hour treatment with CT-1 at 10ng/ml which is the same concentration that was used in all treatments for cell survival assays and westerns. Lanes 3 and 4 contained adult sensory neurons treated with water only and CT-1 respectively. Lanes 5 and 6 contained neonatal sensory neurons treated with water only and CT-1 for 24 hours before protein extraction. Lanes 7 and 8 contained adult sensory neurons treated with water only and CT-1 for 24 hours before protein extraction. The cells were cultured in exactly the same way as the cell survival and cell death experiments and were left for 24 hours before treatments were begun.

As changes in the levels of heat shock proteins were not sufficient to explain the protective effect of CT-1, chemical inhibitors were used to investigate which signalling pathways were involved in the effect of CT-1 in neuronal cells. PD 98059 inhibits the p42/44 MAPK pathway, SB 203580 inhibits the p38 MAPK pathway and LY 294002 inhibits the PI-3 Kinase pathway. PD 98059 blocked the protective effect of CT-1, figure 3.11, demonstrating that activation of the p42/44 MAPK pathway was necessary for the protective effect of CT-1 in neonatal sensory neurons. SB did not block the protective effect suggesting that the p38MAPK pathway was not involved in mediating the protective effect of CT-1. LY is harder to interpret because it was delivered to the cells dissolved in ethanol and the ethanol appeared to have a protective effect in addition to the effect of CT-1. LY reduced survival compared to ethanol but not compared to CT-1. It appears therefore to block the protective effect of ethanol because it reduced the survival in cells treated with ethanol and CT-1 to the level of cells treated with CT-1 alone. However it is not possible to be certain that it is not the other way around, that it was not blocking the effect of ethanol but was blocking the effect of CT-1.

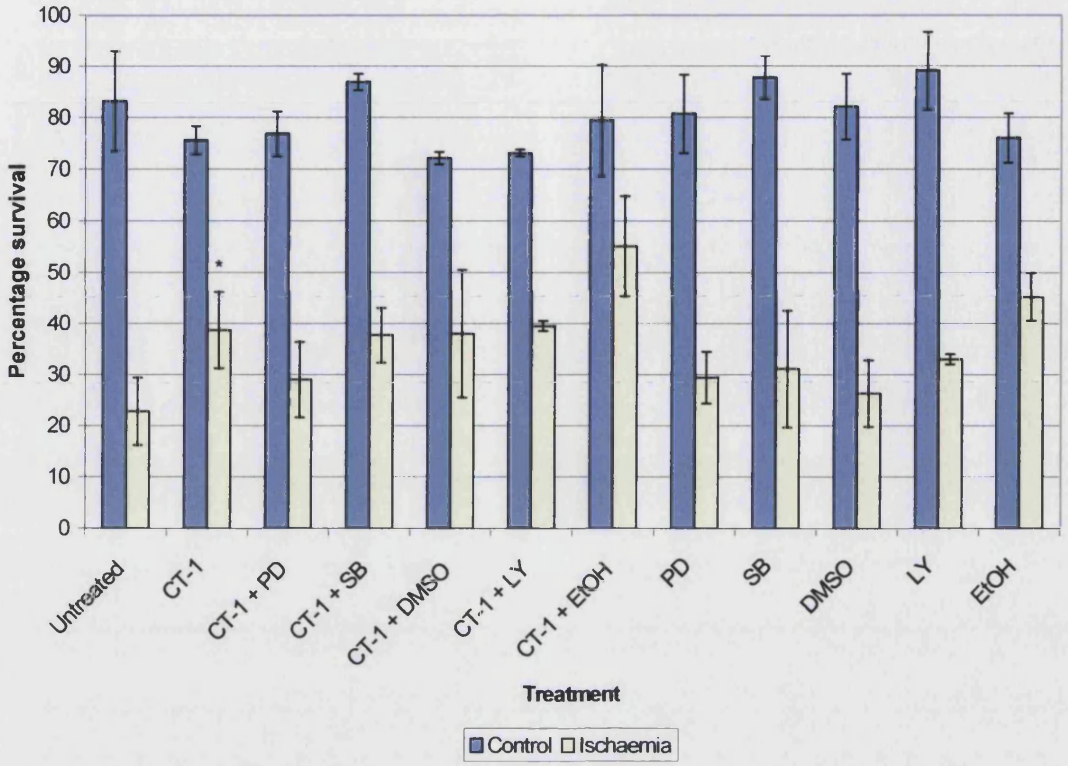
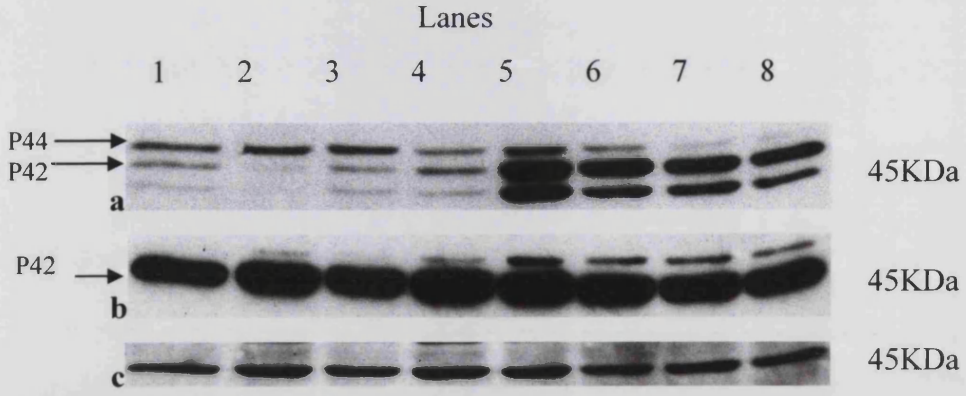


Figure 3.11: The effect of different inhibitors on the protective effect of CT-1 in neonatal sensory neurons. Survival was measured by morphology, cells were pretreated with the inhibitors for 1 hour before the CT-1 treatment. The blue bars represent survival of the control cells and the yellow bars represent survival after ischaemia. PD is PD 98059, SB is SB 203580 and LY is LY 294002. PD 98059 and SB 203580 were dissolved in DMSO and LY was dissolved in ethanol. PD 98059 was used at a final concentration of 50μM, SB 203580 and LY 294 002 were used at a final concentration of 10μM. PD 98059 inhibits p42/22 MAPK, SB 203580 inhibits p38 MAPK and LY 294002 inhibits PI-3 kinase. * $p = 0.002$ CT-1 after ischaemia versus untreated after ischaemia, untreated cells were treated with water and media only, $n = 4$, the error bars represent the standard error of the mean.

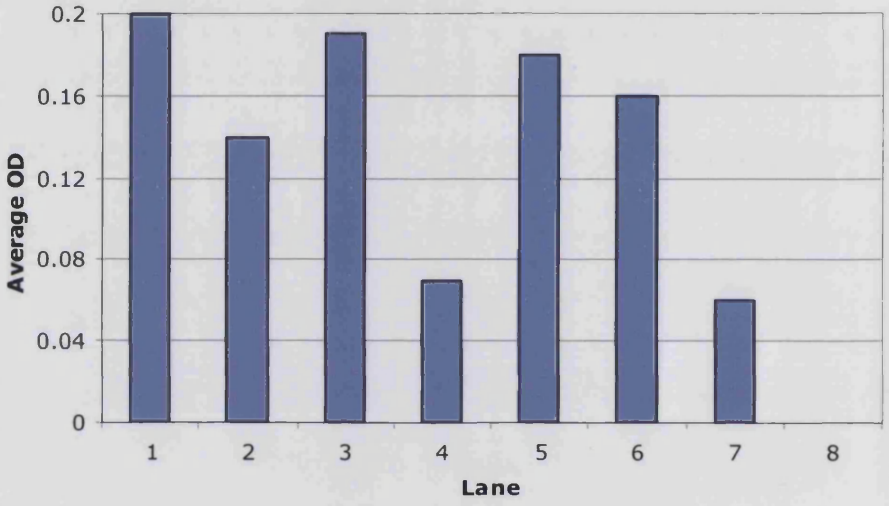
Having shown with chemical inhibitors that p42/44 MAPK was important for the protective effect of CT-1, western blots were used to look at whether CT-1 increased the amount of active (phosphorylated) p42/44 MAPK. The western blot shown in figure 3.12a appears to show that CT-1 did induce a small increase in the amount of active ERK1 (p44 MAPK) in the neonatal cells, (lane 3 versus lane1, blot a) confirming that the p42/44 MAPK pathway was mediating the protective effect of CT-1, however once normalized by β-actin for differences in protein loading and transfer it was clear that this was not the case (figure 3.12d). There was also a decrease in the level of active ERK1 in the adult cells after CT-1 treatment, (lane 7 versus lane 5, blot a and graph e). There was no increase in the level of active p42 MAPK (ERK2) upon CT-1 treatment either, figure 3.12a and e. However, the level of p42 MAPK (ERK2) activation in the adult cells was much higher than that in the neonatal cells perhaps suggesting that CT-1 does not protect adult cells because the pathway is already active in adult cells *in vitro*. Two bands were expected for the active ERK blot but the three bands observed here have been reported previously (Craig et al., 2001). The comparison is between cells treated with CT-1 (Lanes 3 and 7) and cells treated with water (Lanes 1 and 5) because CT-1 was dissolved in water.

In conclusion CT-1 protects neonatal sensory neurons against hypoxic ischaemic stress and this may occur through the p42/44 MAPK (ERK) pathway, CT-1 fails to protect adult sensory neurons against hypoxic ischaemic stress despite the presence of mRNA for the receptor components and an increase in the levels of HSP 70 and 90 upon treatment.



Treatment:	water	ethanol	CT-1	UCN	water	ethanol	CT-1	UCN
Age:	Neonatal				Adult			

d) p44 MAPK (ERK1)



e) p42 MAPK (ERK2)

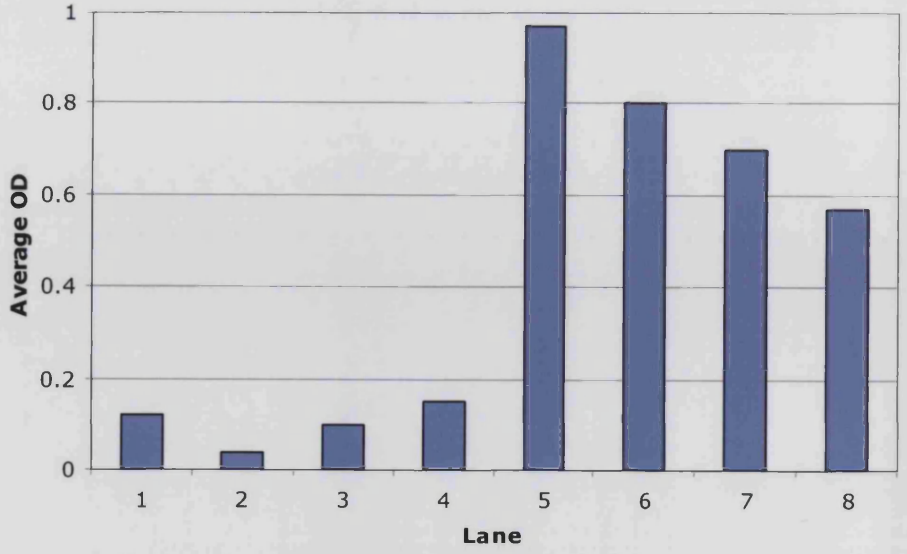


Figure 3.12: p42/44 MAPK protein expression in neonatal and adult sensory neurons treated with CT-1. a, b and c are the same western blot reprobed with antibodies to a) active ERK, b) ERK 2 total protein and c) β-actin. The cultures were set up in the same way as all other experiments and as described in the materials and methods, they were left for 24 hours before treatments were begun. The cells were harvested after 1 hour of each treatment. Amersham Bioscience high molecular weight rainbow markers were used and the nearest molecular weight marker for each antibody is indicated on the right side of the blots, the position of the p42 and p44 MAPK bands are indicated on the left. Lanes 1 – 4 contained protein extracted from neonatal sensory neurons treated with water only, ethanol, CT-1 and UCN respectively. Lanes 5 – 8 contained protein extracted from adult sensory neurons treated with water only, ethanol, CT-1 and UCN respectively. d) is a graph showing the average optical density for each of the active p44 MAPK (ERK1) bands, shown in a, normalized for total protein by β-actin, shown in c. e) is a graph showing the average optical density for each of the active p42 MAPK (ERK2) bands, shown in a, normalized by total ERK2, shown in b. Equal amounts of total protein were initially loaded onto the gel in each lane, differences in the protein levels of the control bands (b and c) may therefore represent differences in the transfer of the proteins from the gel to the membrane. A Bio Rad GS-800 calibrated densitometer with Quantity-one software was used to measure the average OD (optical density) and normalize the protein levels by the control bands.

Ethanol and UCN treatments were included in figure 3.12 but are not relevant to this chapter, they will be discussed in chapter 4.

3.7 Discussion

This chapter has shown that CT-1 has a significant protective effect against hypoxic ischaemia in neonatal sensory neurons, figures 3.5 – 3.7 and 3.11. This is the first time that CT-1 has been shown to protect neurons against ischaemic stress.

The experimental design of the survival assay was such that the trophic effect reported by Thier et al (1999a) after 50 hours in culture was controlled for in the survival assays by taking the first measurement of cell survival after 48 hours and using this as the baseline to compare to the survival after stress. Any death in the cells not treated with CT-1 was excluded from the analysis and therefore could not explain the higher level of survival in the CT-1 treated cells. NGF was included in all the neonatal cultures to support the cells as they are dependent on the presence of a trophic factor in culture, this limited any death from the absence of CT-1 in the untreated cells.

The TUNEL assay also showed a higher survival in the cells treated with CT-1, but it was not statistically significant, figure 3.6. The way that the TUNEL assay was designed also means that you would expect to see a greater amount of death in the apoptosis assay than in the survival assay and this was indeed the case for the controls. The TUNEL assay detects DNA fragmentation so as long as the cells are still physically present and the DNA has not been completely degraded, they will be positively stained by the TUNEL assay. Therefore you would expect a higher level of cell death to be detected in the TUNEL assay than in the survival assay because a certain amount of death occurs after neurons are cultured, the neurons have to be axotomized in order to remove them from the organism and the process of culturing the cells is therefore quite stressful. However there is not substantially more death in the stressed cells in the TUNEL assay than in the survival assay. 4 hours is a relatively early time point, it was chosen because at later time points the cells had degraded enough to detach from the coverslip during the washing steps of the TUNEL assay. It is possible that this time point was too early to detect all the death that occurs as a result of the ischaemic stress, especially since a lot of the damage in other cell types occurs during the recovery phase. If some of the cells were in the early phases of apoptosis they would not be detected by the TUNEL assay because the TUNEL assay detects DNA fragmentation which is a late event in

apoptosis. Therefore if there is more death occurring the full protective effect of CT-1 may not be observed by the TUNEL assay until a later time point. Since the controls appeared to show the trophic effect of CT-1 that has previously been reported by Thier et al (1999a) it is possible that it is only the trophic effect that is detected in this assay and that a later time point or an assay of early apoptotic events would be needed to observe the same protective effect seen in the survival assay. The small time window for identifying TUNEL *in vivo* discussed by Gavrieli *et al* (1992) is due to the loss of the cells to phagocytes, which are not present *in vitro*, hence it would be possible to use a later time point.

The protective effect observed in figure 3.5 was not specific to the culturing conditions and was therefore not caused by a deficiency in the media. Significant protection was also seen in the same cells cultured in different media, figure 3.7, showing that the effect was robust and reproducible.

Surprisingly CT-1 did not protect adult sensory neurons against stress, figure 3.8. Since the culturing conditions had been shown not to affect the protective effect of CT-1 the treatment in adult cells was only carried out in one set of culturing conditions.

The next stage was to examine the mechanism of protection in neonatal cells and try to establish why adult cells were responding differently. The mRNA for the receptor components was present in both neonatal and adult cells, figure 3.3.9, however the protein levels may have been different. The western blots of the heat shock proteins in CT-1 treated cells, figure 3.10, suggested that the initial response to CT-1 by the adult and neonatal cells was different. There was an increase in the levels of HSP 70 and 90 in the adult cells after 1 hour but no increase in HSP 70 or 90 in the neonatal cells (figure 3.12).

It is not surprising that the neonatal neurons expressed gp130 and LIFR mRNA because neonatal dorsal root ganglia sensory neurons have previously been shown to express LIFR mRNA before and after axotomy (Scott et al., 2000) and the distribution of gp130 in the mammalian nervous system is widespread. Also the gp130 and LIFR knockout mice had neuronal abnormalities and in this chapter it has been shown that CT-1 is able

to influence the survival of neonatal sensory neurons, which suggests that the receptor components are likely to be expressed and available on the plasma membrane.

It is perhaps more surprising that the adult cells expressed gp130 and LIFR mRNA since the CT-1 treatment did not affect the survival of the cells. However the adult cells were clearly capable of responding to CT-1 because the CT-1 treatment caused an increase in the levels of the heat shock proteins HSP 70 and HSP 90. Adult sensory neurons have previously been shown to express gp130 and LIFR protein (Gardiner et al., 2002). All of the adult dorsal root ganglia sensory neurons examined express gp130 in the cytoplasm and on the plasma membrane, this is not affected by axotomy. LIFR, although expressed, is confined to the nuclear compartment. 6 hours after axotomy there is punctate cytoplasmic LIFR which remains for 14 days. Small neurons, those positive for the neuropeptide CGRP or the surface marker isolectin B4 which are markers of small diameter peptidergic neurons and small non-peptidergic neurons respectively, express proportionally more cytoplasmic LIF. The total amount of LIFR does not increase and the level of protein in the nuclear compartment decreases which suggests that it translocates to the cytoplasm. It is possible that it is moved to the plasma membrane and then internalized as part of the complex, perhaps enough LIFR is expressed on the plasma membrane for CT-1 to bind and cause the resulting increase in HSP 70 and HSP 90 expression. Perhaps there is too little expressed on the plasma membrane for CT-1 to cause the downstream signalling necessary to influence the survival of the cells, this seems unlikely since binding of ligands to receptor complexes does not usually result in the activation of one signalling molecule but many, normally resulting in amplification of the signal. Clearly more work is needed if the lack of response of adult sensory neurons to CT-1, compared to neonatal sensory neurons, is to be understood.

The levels of heat shock proteins in the adult cells had dropped again after a 24 hour exposure to CT-1, which is when the stress was administered, explaining why the increase in heat shock proteins itself was not protective in the adult cells. The level of HSP 27 was higher in the adult cells than the neonatal cells as would be expected. HSP 27 expression is developmentally regulated and increases gradually between embryonic day 15 and postnatal day 21 when adult levels are reached (Costigan et al., 1998). The expression of HSP 27 is upregulated dramatically after peripheral nerve injury and

reaches progressively higher levels in P2, P7, P21 and adult rats (Costigan et al., 1998; Lewis et al., 1999).

CT-1 treatment led to increases in HSP 90 in cardiac myocytes and possibly HSP 70 (Stephanou et al., 1998a; Railson et al., 2000). It is possible that if the adult cells had been stressed after a 1 hour treatment the increase in heat shock proteins would have been protective. However in cardiac cells CT-1 was shown to increase the level of HSP 90 protein, as did heat shock, but CT-1 treatment and heat shock together resulted in a higher level of HSP 90 than CT-1 treatment but a lower level than that induced by heat shock alone (Railson et al., 2000). CT-1 is protective against heat shock in cardiac cells which suggests that it may in some way prevent the cells from becoming too stressed. The lack of heat shock protein induction in neonatal sensory neurons is interesting because it represents a divergence of the effect of CT-1 in neurons from that seen in cardiac cells even though CT-1 is protective in both systems. The lack of protection in the adult cells is also a key difference between the effect of CT-1 in cardiac cells and in neurons. CT-1 protected both adult intact hearts *ex vivo* and adult, as well as neonatal, cardiac myocytes *in vitro* against simulated ischaemia reperfusion when used to treat the cells before ischaemia and at reperfusion (Liao et al., 2002).

Since an increase in heat shock protein levels could not explain the protective effect in neurons, chemical inhibitors were used to establish which signalling pathway was being activated by CT-1. The cell survival experiments, figure 3.11, suggest that CT-1 activated the p42/44 MAPK pathway in neonatal sensory neurons and that this pathway was responsible for the protective effect, however, this was not supported by an increase in activated p42/44 MAPK in the western blot, figure 3.12. The p42/44 MAPK pathway is responsible for the protective effect of CT-1 in cardiac cells. In cardiac cells the PI-3 kinase pathway is also important (Brar et al., 2001b; Liao et al., 2002) but it is not possible to conclude with any certainty whether LY, the PI-3 kinase inhibitor, had an effect on the level of protection due to CT-1 in sensory neurons because the vehicle for delivering LY (ethanol) also had a protective effect. These results suggest a possible reason for the lack of protection in adult neurons. A greater amount of the p42 MAPK (ERK2) protein was phosphorylated in untreated adult cells than neonatal cells, figure 3.12a and e, suggesting that CT-1 could not protect those cells because the pathway was

already active. It is interesting to note that the level of survival after the same stress was no higher in the untreated adult cells than it was in the untreated neonatal cells (figures 3.7 and 3.8) despite the higher level of active (phosphorylated) p42 MAPK (ERK2) in the adult cells.

In conclusion CT-1 protects neonatal sensory neurons against hypoxic ischaemic stress, possibly through the p42/44 MAPK pathway, but does not protect adult sensory neurons against the same stress. The protective effect of CT-1 was not mediated by heat shock proteins, therefore CT-1 could not be used as a non-stressful way to induce heat shock proteins in these cells. UCN is another peptide that has been shown to protect cells against stress through the p42/44 MAPK pathway but it also utilizes the PI-3 Kinase and PKA pathways (Brar et al., 2000; Schulman et al., 2002; Pedersen et al., 2002; Brar et al., 2002b). UCN has been shown to induce expression of HSP 90 in cardiac myocytes (Brar et al., 2002a) and it is a neuropeptide, so it was postulated that UCN could provide an alternative way to induce endogenous expression of heat shock proteins in a non-stressful way in neurons that might protect sensory neurons against stress induced damage.

Chapter 4: The effect of urocortin in neonatal and adult sensory neurons.

4.1 Introduction

Urocortin (UCN), a peptide related to corticotrophic releasing factor (CRF), is a ligand for corticotrophic releasing factor receptors 1 and 2 (CRFR1 and CRFR2). UCN is predominantly expressed in the midbrain, see 1.5, but there are also UCN immunoreactive fibres the length of the spinal cord (Bittencourt et al., 1999).

Messenger RNA for UCN and two of the CRFR2 isoforms, CRFR2α and CRFR2β, are present in peripheral tissues including the heart (Kimura et al., 2002). Only the CRFR2 receptor is present in the heart and CRFR2 knockout mice exhibit elevated basal blood pressure and an impaired cardiovascular response to UCN (Coste et al., 2000; Bale et al., 2000). Expression of urocortin mRNA increases in response to hypoxic ischaemia in cardiac myocytes (Okosi et al., 1998), and UCN is secreted into the media by both cardiac myocytes and cardiac non-myocytes in neonatal cultures *in vitro* (Brar et al., 1999a; Ikeda et al., 2002). Exogenously applied UCN was first shown to protect neonatal cardiac myocytes against simulated ischaemia *in vitro* by Brar et al (1999a), and also protects adult cardiac myocytes against simulated ischaemia *in vitro* and whole adult hearts *ex vivo* (Brar et al., 2000; Scarabelli et al., 2002; Gordon et al., 2002).

The protective effect of UCN is mediated by the p42/44 MAPK pathway, via CRFR2, in both cultured cardiac myocytes and whole hearts *ex vivo* (Brar et al., 2000; Schulman et al., 2002). Activation of the AKT (PI-3 Kinase) pathway is also essential for the protective effect of UCN (Brar et al., 2002b).

Interestingly UCN is also expressed in cardiac non-myocytes (Nishikimi et al., 2000) and exogenous UCN increases cell proliferation in these cells which may provide further benefits after ischaemia *in vivo*. This appears to be mediated, at least in part, by a cAMP dependent protein kinase A pathway but the CRF receptor 2 appears not to be

necessary since the effect is not blocked by the CRFR competitive antagonist astressin (Ikeda et al., 2002).

UCN has a protective effect in the heart through the p42/44 MAPK pathway and in chapter 3 it was shown that this MAPK pathway mediated a protective effect in sensory neurons against simulated ischaemia. The protective effect of UCN against ischaemia has only been studied in the heart and UCN treatment in cardiac myocytes increased the level of HSP 90 (Brar et al., 2002a).

In cultured hippocampal cells UCN protects against oxidative and excitotoxic insults (Pedersen et al., 2002). UCN has also been shown to inhibit oedema caused by thermal injury in rats (Turnbull et al., 1996). The effect of UCN on cells from the peripheral nervous system has not been investigated.

It was therefore proposed that UCN might act in a non-stressful manner to induce endogenous heat shock protein expression and protect sensory neurons from ischaemia. In the heart UCN activated the MAPK pathway that had been shown to mediate the protective effect of CT-1 in sensory neurons. However, CT-1 failed to protect sensory neurons from adult rats, hence it was proposed that UCN might provide an alternative method to elevate endogenous HSP expression in sensory neurons and might therefore protect adult sensory neurons. In this chapter UCN was tested for a potential protective effect against simulated hypoxic ischaemia in dorsal root ganglia sensory neurons from both neonatal and adult rats.

4.2 Methods

The experiments in this chapter were carried out as described in chapter 3. The control cells were treated with the same volume as UCN of 100% ethanol as ethanol was used as the vehicle to deliver the UCN.

4.3 The effect of UCN in neonatal sensory neurons

UCN was predicted to protect neonatal sensory neurons against simulated ischaemia because in the heart it stimulates the same pathway as CT-1 and CT-1 is protective in sensory neurons, chapter 3. The neonatal sensory neurons were treated with UCN for 24 hours. However, as can be seen in figure 4.1 this did not result in protection against simulated ischaemia.

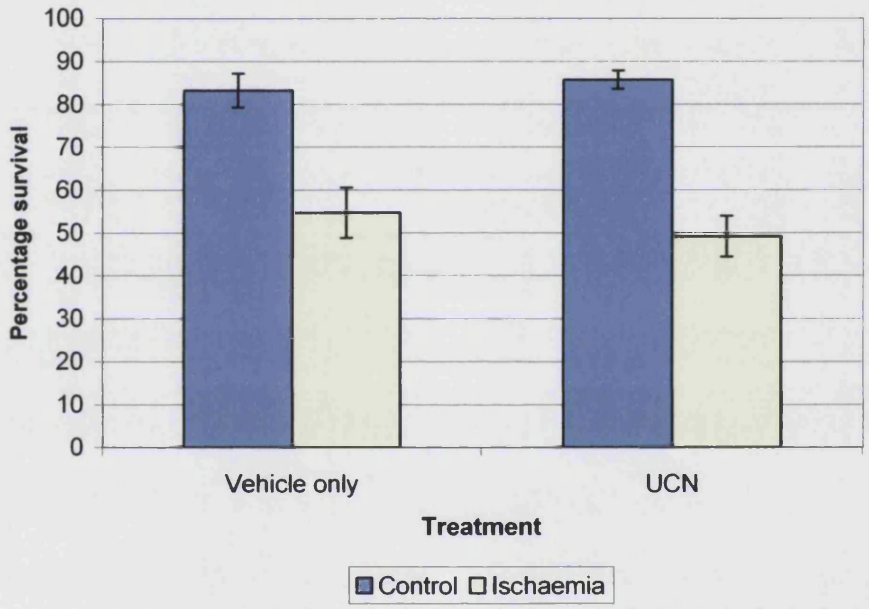


Figure 4.1 Urocortin does not protect neonatal sensory neurons against a hypoxic ischaemic stress. Cells were cultured in DMEM and survival was measured by morphology as described in the methods, error bars represent the standard error of the mean, n = 11.

It is possible for UCN to protect against apoptosis but for the cells to die by necrosis instead so TUNEL was used as an assay of apoptosis to test whether the death occurring was apoptotic and whether UCN was having an effect on the level of apoptosis in the neonatal cells, figure 4.2.

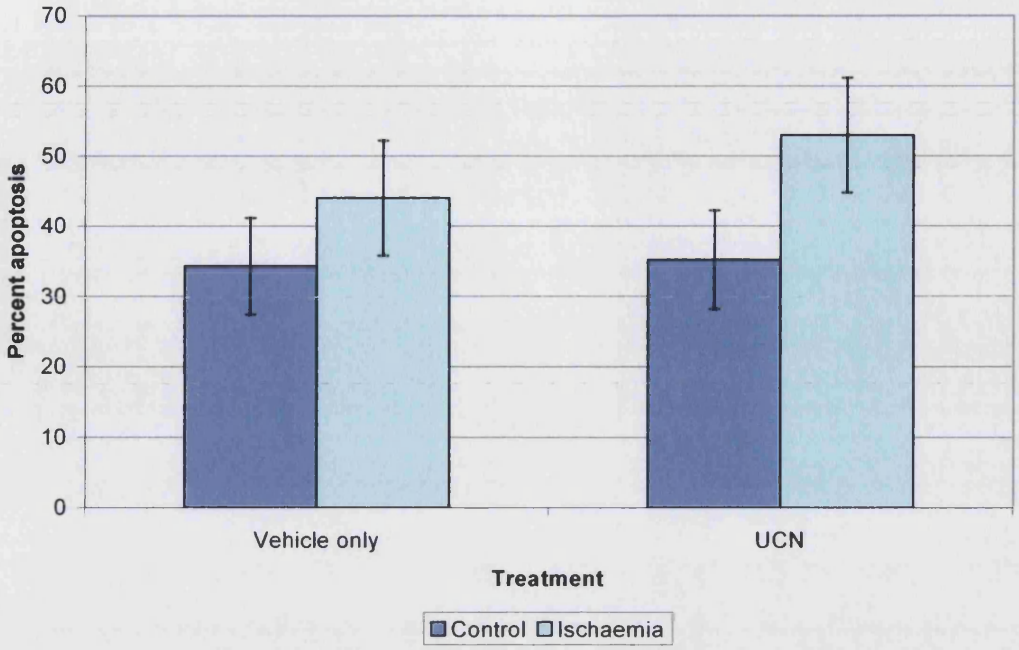


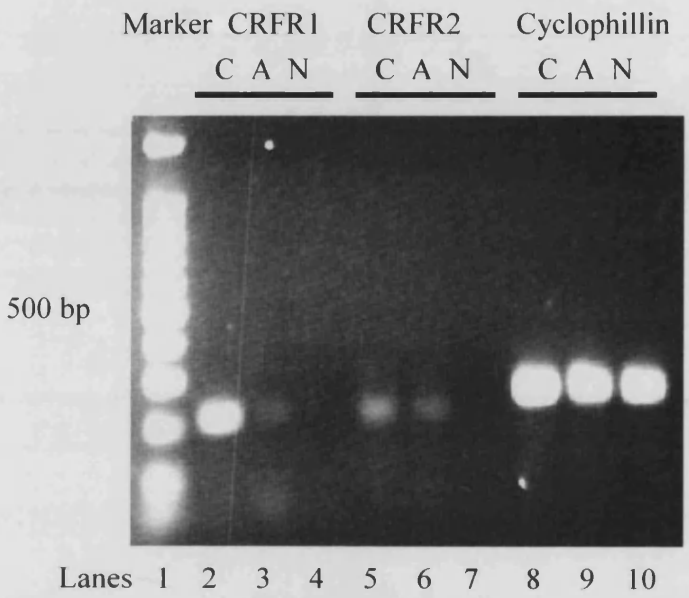
Figure 4.2 Urocortin does not protect neonatal sensory neurons against a hypoxic ischaemic stress as measured by TUNEL. Cells were cultured in DMEM and the TUNEL assay was carried out as described in the methods, error bars show the standard error of the mean, n = 11.

Figure 4.2 confirms that the death induced by this model of simulated ischaemia was apoptotic, in the survival assay 50% of the UCN treated neurons survived and in the TUNEL assay 50 % of the UCN treated neurons were apoptotic. In the neurons treated with the vehicle only (EtOH) 55 % of the cells survived and 45% were apoptotic. The Tunel result shows that the level of death was not reduced by treatment with UCN and therefore supports the result shown in figure 4.1 that UCN does not have a protective effect in neonatal sensory neurons against simulated ischaemia.

4.4 The UCN receptor

Since UCN did not have a protective effect in the neonatal cells RT-PCR was performed to identify the presence or absence of mRNA for the two receptors CRFR1 and CRFR2. Figure 4.3 shows the PCR product run out on an agarose gel and stained with ethidium bromide as described in the materials and methods. RNA extracted from adult rat brain tissue was used as a positive control for both CRFR1 and CRFR2 because both have been shown to be present in rat brain tissue (Perrin et al., 1993; Lovenberg et al., 1995b).

A


Marker
CRFR1
CRFR2
Cyclophillin
C A N
C A N
C A N
500 bp
Lanes 1 2 3 4 5 6 7 8 9 10

B

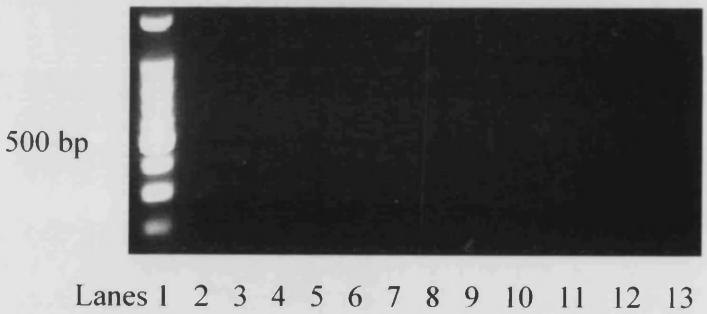

500 bp
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 4.3 The mRNAs for the UCN receptors CRFR1 and CRFR2 are expressed in adult but not neonatal sensory neurons. A: Lane 1 contained a promega 100bp ladder, lane 2 contained the product of the reaction to detect CRFR1 in the adult brain which was used as a positive control (Labelled C). Lane 3 contained the reaction product for CRFR1 in adult sensory neurons (Labelled A) and lane 4 the product for the CRFR1 reaction in neonatal sensory neurons (Labelled N). A small amount of product can be seen in lane 3 but no product was visible in lane 4 even when more product was loaded onto the gel and maximum exposure was used, data not shown. Lane 5 contained the positive control for the CRFR2 reaction from adult brain (Labelled C). Lane 6 shows the product for the CRFR2 reaction in adult sensory neurons (Labelled A) and lane 7 the result of the reaction to amplify CRFR2 from neonatal sensory neurons (Labelled N). Lane 8 contained the reaction product for cyclophilin amplified from the adult brain (Labelled C). Lanes 9 and 10 contained the product for cyclophilin amplified from the adult and neonatal sensory neurons respectively Labelled A and N respectively). The cyclophilin reactions were all done on the same RT reaction as the CRFR1 and CRFR2 reactions. The sensory neurons were cultured in the same conditions used for the experiments and then the RNA was extracted for the positive controls RNA was extracted from a piece of brain tissue frozen in liquid nitrogen as soon as it was removed. All product sizes matched the predicted sizes, see table 2.5.

B: Control samples. Lane 1 contained the same promega 100bp ladder, lanes 2 – 4 contain reactions done with minus RT controls for adult brain, adult sensory neurons and neonatal sensory neurons respectively with the CRFR1 primers. Lane 5 contained a no template reaction for CRHR2, the same ddH_2O used to set up all the reactions was used as a template for this reaction. Lanes 6 – 9 contained reactions done with minus RT controls for adult brain, adult sensory neurons and neonatal sensory neurons respectively with the CRFR2 primers. Lane 10 contained a no template reaction for CRHR2, the same ddH_2O used to set up all the reactions was used as a template for this reaction. Lanes 10-12 contained the minus RT controls for cyclophilin, from adult brain in lane 10, adult sensory neurons lane 11 and neonatal sensory neurons lane 12. As for the CRFR1 and CRFR2 rections lane 13 contained a water only control for the cyclophilin primers. For both gels 5µl of a 25µl reaction was run on the gel.

No RT-PCR product was detected from the neonatal sensory neuronal cells for CRFR1 or CRFR2, although there were reaction products for the CRF receptor positive controls and the cyclophillin positive control for the neonatal RNA, figure 4.3. This suggests that they do not express either of the receptors for UCN and would explain why UCN treatments did not have a protective effect on the survival of the cells after simulated ischaemia, figures 4.1 and 4.2. However, figure 4.3 also shows that the mRNA for both of the UCN receptors was present in the adult cells. Only low levels of RT-PCR product were detected but this was not a quantitative reaction. No products were produced from the minus RT controls or the no template controls showing that there was no contamination, only single bands of the correct predicted size resulted from the PCR reactions showing that there was no amplification from genomic DNA. The primers were designed to produce much larger products if there was amplification from the genomic DNA.

Since a product for receptor mRNA was detected for the adult cells, suggesting that they do express the UCN receptors, and CT-1 had an age dependent effect, a 24 hour UCN treatment was used to investigate whether UCN had a protective effect in adult sensory neurons.

4.5 The effect of UCN in adult sensory neurons

Figure 4.4 shows that UCN did not have a protective effect on adult sensory neurons subjected to simulated ischaemia despite the mRNA for both potential receptors being present in these cells.

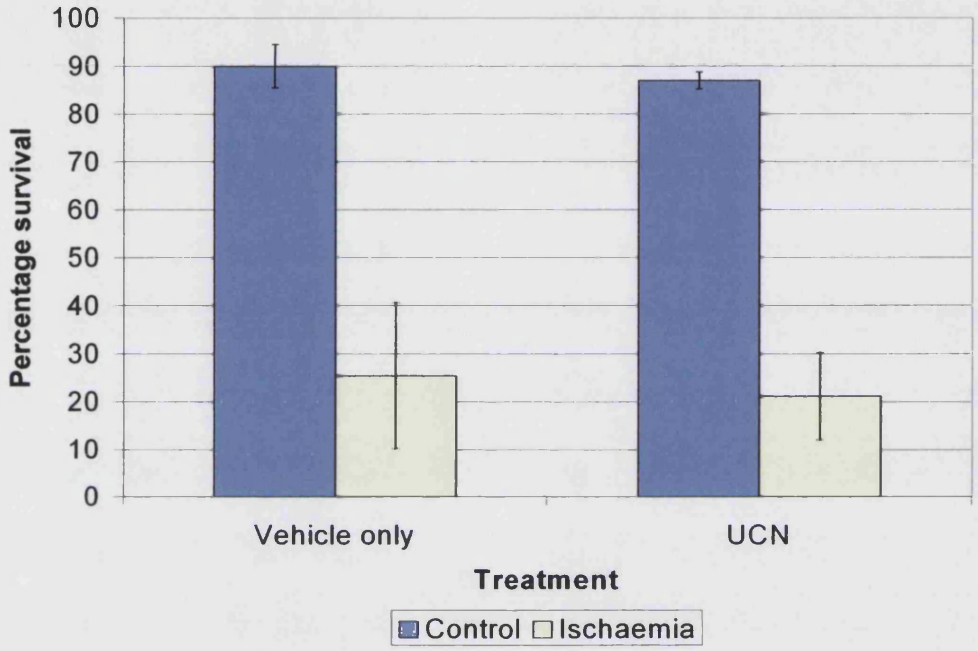


Figure 4.4: Urocortin does not protect adult sensory neurons against a hypoxic ischaemic stress. Cells were cultured in DMEM and survival was measured by morphology, error bars represent the standard error of the mean, n = 3.

Alternative culturing conditions were used to investigate whether the culturing conditions affected the result, figure 4.5. However UCN did not protect cells cultured in the alternative media F14.

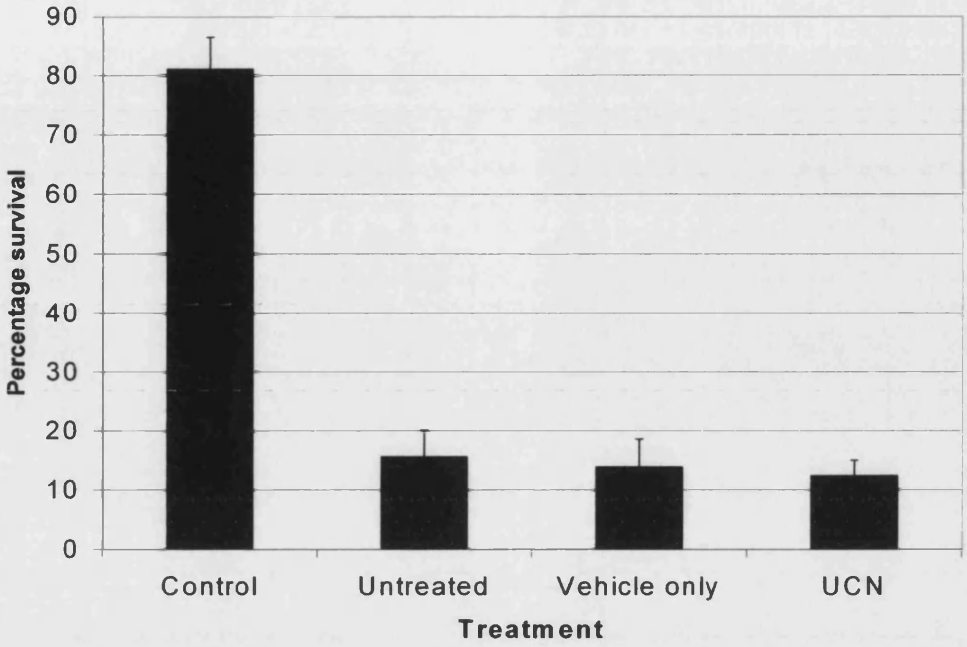


Figure 4.5: Urocortin does not protect adult sensory neurons cultured in F14 media against hypoxic ischaemia. Cells were cultured in F14 with serum, the error bars represent standard error of the mean, n = 2.

A western blot was carried out to investigate whether UCN treatment activated the p42/44 MAPK pathway as it did in the heart. Figure 3.12, see chapter 3 (page 102), is a western blot probed with phosphorylated (active) p42/44 MAPK, unphosphorylated (inactive) p42 MAPK and β-actin. It shows higher levels of active p44 MAPK in both the neonatal and adult sensory neurons, and a higher level of p42 MAPK in the adult cells, treated with ethanol (Vehicle only) than UCN but curiously a higher level of p42 MAPK in the neonatal cells treated with UCN than ethanol. P44 MAPK was normalized by β-actin and p42 MAPK was normalized by total p42 MAPK. In conclusion, UCN only activated the p42 MAPK pathway in the neonatal sensory neurons even though both the neonatal and adult sensory neurons express the mRNA for both UCN receptors, which may explain why it did not protect adult sensory neurons against stress.

4.6 Discussion

This chapter has shown that UCN does not have a protective effect in neonatal or adult sensory neurons. This model of simulated ischaemia did cause enough cell death to detect a protective effect if protection had occurred, there was a 30 – 40% drop in survival in the neonatal cells after stress and a 10 – 20% increase in apoptosis in the neonatal cells (Figures 4.1 and 4.2). In the adult cells there was a 60 – 70% drop in survival after stress (figures 4.4 and 4.5). The cell death caused was almost all apoptotic, figures 4.1 and 4.2, because the survival and apoptosis levels account for approximately 100% of the cells counted between them. Since all the cells appear to be accounted for it can be concluded with some certainty that there is not a protective effect. Figures 4.4 and 4.5 show that the culturing conditions used had no effect on the result, so the lack of protection by UCN is not due to an anomaly in the culturing conditions.

The lack of any mRNA for either of the UCN receptors in the neonatal sensory neurons explains why no protective effect was observed in neonatal sensory neurons. If there are no receptors present on the cell membrane then the UCN would be unable to initiate any signalling cascades and there would be no downstream effects. This was not a quantitative reaction so no conclusions can be drawn about the relative amounts of mRNA in the samples, it is also important to note that it does not necessarily follow that because there is mRNA present in cells they are expressing the protein. However it seems unlikely that the receptors are expressed since there is no detectable mRNA and there is no protective effect, figure 4.3.

In the adult sensory neurons there was detectable mRNA suggesting that the cells may express both of the UCN receptors. The amount of PCR product from both CRFR1 and CRFR2 was very low compared to the controls, one explanation for this could be that there was very little mRNA present to begin with, figure 4.3. It is possible that the CRFR2 reaction was not very efficient because the control sample yielded only a small amount of reaction product although it is also possible that there was only a low level of mRNA for the CRFR2 present in the brain sample used. Although the mRNA for the

receptors was present in the adult cells this is no guarantee that the protein was expressed and this may be one possible explanation of why UCN did not protect adult sensory neurons from stress.

P42/44 MAPK is involved in the protective effect of UCN in both hippocampal cells and cardiac myocytes (Brar et al., 2000; Schulman et al., 2002; Pedersen et al., 2002; Brar et al., 2002b). Only slight activation of p42 MAPK was observed in the UCN treated neonatal sensory neurons, figure 3.12, but there was no activation of p44 MAPK in either age group or p42 MAPK in the adult cells. This may be because there was no functional receptor present for the UCN to bind to in the adult cells, or because UCN bound to the receptors in adult sensory neurons but the binding did not result in the necessary signalling pathways being activated. The level of activated p42 MAPK, as observed in chapter 3, was much higher in the adult sensory neurons than the level of activated p42 MAPK in the neonatal sensory neurons. Hence, it is possible that the UCN is unable to increase the activation of the p42 MAPK in adult cells because it has already been activated by something else, i.e. the culturing process, or the level of p42 MAPK activation may be constitutively higher in adult cells.

A complicating factor in the lack of protection by UCN is that UCN was delivered to the cells in ethanol. Ethanol appeared to have a protective effect when used alone in neonatal sensory neurons cultured in DMEM, see figure 3.11. Interestingly, ethanol did not appear to have a protective effect in F14 media, in figure 4.5. an untreated well was included as well as an ethanol only (Vehicle only) control and this showed that compared to untreated cells neither UCN nor ethanol had an effect. In fact, UCN appeared to have a death inducing effect, although this was not statistically significant, in neonatal and adult sensory neurons cultured in DMEM, it decreased cell survival (figures 4.1 and 4.4), increased cell death (figure 4.2) and decreased p44 MAPK activity in neonatal and adult sensory neurons and p42 MAPK activity in adult sensory neurons (figure 3.12). It is possible that because ethanol affects cell survival in neurons cultured in DMEM that it is interfering with the effect of UCN. However, since the cells treated with UCN are also being treated with ethanol the only way this is possible is if UCN antagonizes the ethanol because the cells treated with both UCN and ethanol do not survive as well as the cells treated with ethanol alone.

In conclusion treatment with UCN did not result in any detectable protection of sensory neurons from neonatal or adult rats against a hypoxic ischaemic stress. UCN treatment did not significantly activate the p42/44 MAPK pathway but the induction of heat shock proteins was not examined because there was no protective effect.

Chapter 5: The protective effect of heat shock proteins in different age groups

5.1 Introduction

There is an age related decline in the heat shock response both *in vitro* and *in vivo* as discussed in chapter 1. This impairment reduces the cells', and therefore the organisms', ability to survive stressful conditions. The loss of heat shock protein inducibility has also been observed in the heart and there is a corresponding loss of preconditioning (Abete et al., 1996; Locke and Tanguay, 1996; Tani et al., 1997; Fenton et al., 2000; Schulman et al., 2001). Preconditioning is the protective effect of a non-lethal stress against a subsequent lethal stress. It was first described by Murry et al (1986) and is an important mechanism that reduces morbidity and mortality in organisms. It is most commonly investigated in the heart where it has clear clinical relevance (Deutsch et al., 1990; Iwasaka et al., 1994; Abete et al., 1997). It has been observed in aged individuals from all mammalian species investigated including pigs, rats, rabbits, humans and dogs, it has also been shown in Jurkat cells and an immortalized neuronal cell line (Murry et al., 1986; Schott et al., 1990; Deutsch et al., 1990; Liu et al., 1991; Cohen et al., 1991; Liu and Downey, 1992; Yellon et al., 1992; Mailhos et al., 1993; Downey and Cohen, 1997; Samali et al., 1999). The reported loss of preconditioning begins in middle age and is complete in old age (Tani et al., 1997; Schulman et al., 2001). If preconditioning could be restored in vivo there could be some important benefits including reduced surgical morbidity and mortality.

Some research shows that increasing heat shock proteins in replicatively senescent cells can be beneficial. Mild heat shock increases the basal level of HSP 70 and reduces the accumulation of oxidized and glycoxidized proteins in human fibroblasts in vitro (Verbeke et al., 2001). Repeated mild heat shock improves the survival and functional ability of the cells when exposed to a range of stresses. It is accompanied by an increase in HSP 70 in young and old cells, an increase in HSC 70 and HSP 27 in old cells and a decrease in HSP 90 in old cells (Fonager et al., 2002). Repeated mild heat stress delays the onset of age related changes, such as cell enlargement and β-galactosidase expression but does not prolong the life span of the human skin fibroblasts (Rattan,

1998). Enhancing the expression of HSP 70 by using an adenovirus vector protects senescent fibroblasts against heat shock (Volloch et al., 1998).

Since the induction of heat shock proteins by stressful stimuli becomes impaired with age, and increasing expression of the heat shock proteins is beneficial in replicatively senescent cells, the aim of this chapter was to investigate whether aged sensory neurons could be protected against heat shock and ischaemia by heat shock proteins. This has not previously been shown in primary, post mitotic neurons and it is not known whether it is possible to rescue these cells from a lethal stress. To do this, HSV-1 based viral vectors containing the genes for HSP 27, HSP 56 and HSP 70 were used to express exogenous heat shock proteins and therefore increase the total levels of heat shock proteins in neonatal and aged sensory neurons.

The HSV-1 virus vectors used in this chapter were all constructed by Wagstaff (1997). They have previously been used to express the heat shock proteins HSP 27, 70 and 56 in ND7 cells and neonatal sensory neurons. In ND7 cells the HSP 70 virus vector and the HSP 27 virus vector provide protection against a lethal heat shock and a lethal ischaemic stress. HSP 56 does not protect ND7 cells against heat stress or ischaemia. In neonatal sensory neurons infection with the virus vectors demonstrates that HSP 27, HSP 56 and HSP 70 gives protection against heat shock and HSP 27 and HSP 70 protect against simulated ischaemia, HSP 56 provides only slight protection against simulated ischaemia. Only HSP 27 gives significant protection to neonatal sensory neurons against nerve growth factor withdrawl and to ND7 cells from serum withdrawl and retinoic acid induced apoptosis (Wagstaff et al., 1999).

An HSV-1 virus vector containing a constitutively active form of HSF-1 induces HSP 70 but not HSP 27 in ND7 cells and neonatal sensory neurons, and accordingly provides protection against thermal and ischaemic stress but does not protect against serum withdrawl and retinoic acid in ND7 cells or NGF withdrawl in neonatal sensory neurons (Wagstaff et al., 1998b). This virus vector was not used in this thesis as it did not activate a range of heat shock proteins in sensory neurons and therefore would not have contributed to the study more than the HSP 70 virus. It also induces expression of different HSPs in different cell types (Wagstaff et al., 1998a).

5.2 Methods

As for previous experiments the sensory neurons were cultured on day 1 and infected with the virus vectors 24 hours later. The cells were left for a further 24 hours to allow for expression of the transgene and then the cells were subjected to either heat shock or simulated ischaemia. Survival was measured by morphology 24 hours after the stress was administered and the TUNEL assay was carried out 4 hours after the stress. TUNEL was carried out using rhodamine labeled dNTPs instead of the Fluoroscein labeled dNTPs used in previous experiments so that the TUNEL fluorescence could be distinguished from the GFP expression. GFP expression was used to determine the efficiency of infection in the sensory neurons and was observed in a minimum of 75% of the cells in all experiments.

The neonatal cells in this chapter were subjected to three hours of hypoxic ischaemic stress rather than the four hours used for all other experiments. In the virally infected neonatal cells four hours of stress was too stressful and all of the cells died. Time courses of hypoxic ischaemia were carried out previously in both adult and neonatal sensory neurons to determine the appropriate length of time to expose the cells to hypoxic ischaemic conditions, see figures 3.3 and 3.4.

5.3 The use of a virus vector to express higher levels of heat shock proteins

Before using the virus vectors to increase the expression levels of heat shock proteins in sensory neurons it was necessary to verify that infection with the vectors led to expression of the correct heat shock protein in these cells. Figure 5.1 shows that infection with the HSP 27 and HSP 70 virus vectors leads to high levels of expression of the proteins in ND7 cells.

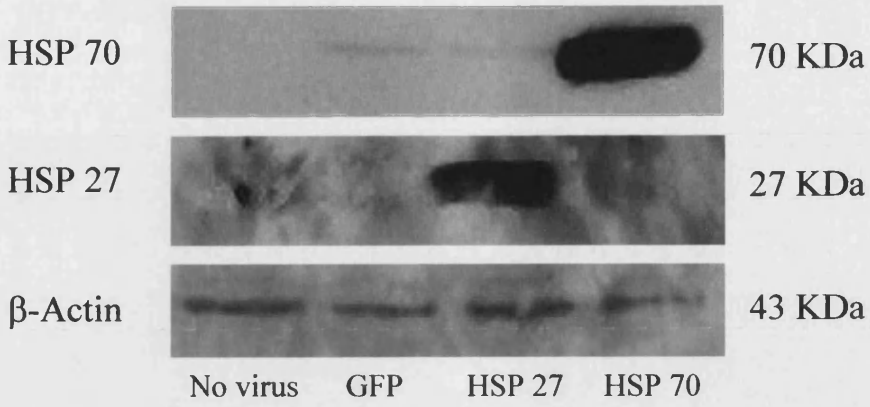


Figure 5.1: Expression of heat shock proteins using the virus vectors in ND7 cells. ND7 cells were either infected with the GFP expressing virus, infected with the HSP 27 expressing virus or infected with the HSP 70 expressing virus or left uninfected. The cells were left for 24 hours after infection to allow expression of the transgene and were then lysed and used for western blotting. The protein levels were equalized using a Bradford assay and the western was probed with β-actin to assess the protein levels. A single western blot was reprobed with antibodies to HSP 27, HSP 70 and β-actin. The blot shows that the virus containing the HSP 27 gene expresses high levels of HSP 27 protein and the virus containing the HSP 70 gene expresses high levels of HSP 70 protein.

Very low levels of HSP 70 appeared to be induced by the virus infection itself as a faint band was present both in the GFP and HSP 27 virus infected cells. However the level of HSP 70 protein expressed by the HSP 70 virus was much greater.

Having demonstrated that cells infected with virus vectors expressed high levels of the correct protein, they were tested in primary cultures of sensory neurons as these were the cells used in all further experiments. The western blots shown in figure 5.2 confirmed that the virus vectors infected adult sensory neurons and that infection resulted in increased expression of the correct heat shock proteins. The cells had previously been stained with a neuron specific antibody to confirm that the cells were sensory neurons, see figure 3.2.

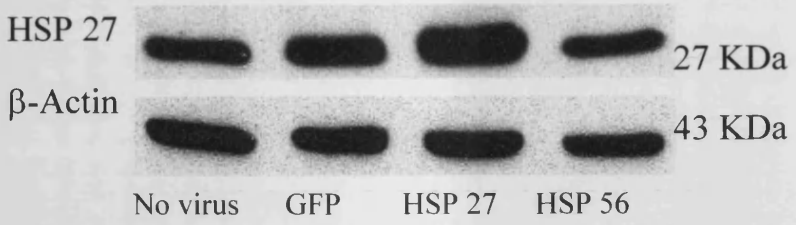


Figure 5.2a: Expression of HSP 27 in adult sensory neurons.

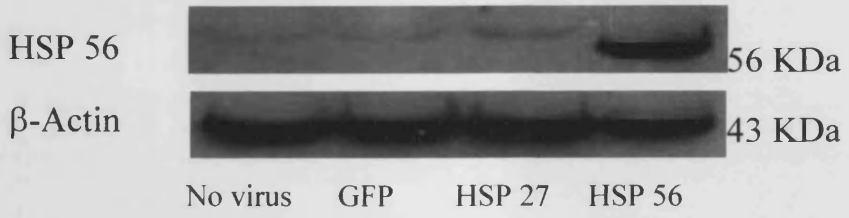


Figure 5.2b: Expression of HSP 56 in adult sensory neurons.

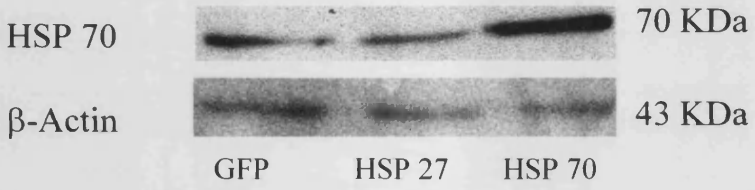


Figure 5.2c: Expression of HSP 70 in adult sensory neurons.

Figure 5.2: Western blots of adult sensory neurons infected with the virus vectors. Adult sensory neurons were either infected with the GFP, HSP 27, HSP 56 or HSP 70 virus vectors or left uninfected. The cells were left for 24 hours after infection to allow for protein expression. The cells were lysed and the level of protein equalized using a Bradford assay before using the lysate for western blotting. Each blot was probed with an antibody to one of the heat shock proteins and then reprobed with β-actin to assess the protein levels. Figure 5.2a, 5.2b and 5.2c show the results for western blots probed with anti-HSP 27 antibody, anti-HSP 56 and anti-HSP 70 antibody respectively. The antibodies used are listed in table 2.4 and the bands were all of the expected size.

The levels of β-actin were approximately equal in all three western blots (figure 5.2). HSP 70 expression was visible in all three virus infected samples on the HSP 70 western blot but was higher in the cells infected with the HSP 70 virus (figure 5.2c). HSP 56 expression was only visible in the cells infected with the HSP 56 virus suggesting that the endogenous levels were very low (figure 5.2b). Endogenous levels of HSP 27 in the cultured cells appeared to be quite high since even the uninfected cells had an obvious band (figure 5.2a), however the level of HSP 27 was still increased by the HSP 27 virus. It is notable that the cells infected with the GFP virus had increased levels of HSP 27 expression, as observed with HSP 70 in ND7 cells, suggesting that the GFP virus had some effect on the cells itself.

Each virus vector contained and expressed only one transgene, so parallel infections were carried out with the same virus vector backbone containing the GFP gene so that the level of infection could be assessed for each experiment. The expression of the GFP was visualized by fluorescent microscopy (figure 5.3). Since no fluorescence was observed in the uninfected cells it was assumed that the fluorescence observed in the infected cells was GFP, GFP antibodies have been used previously to demonstrate that the protein expressed by this virus vector is GFP (Papageorgiou et al., 2002).

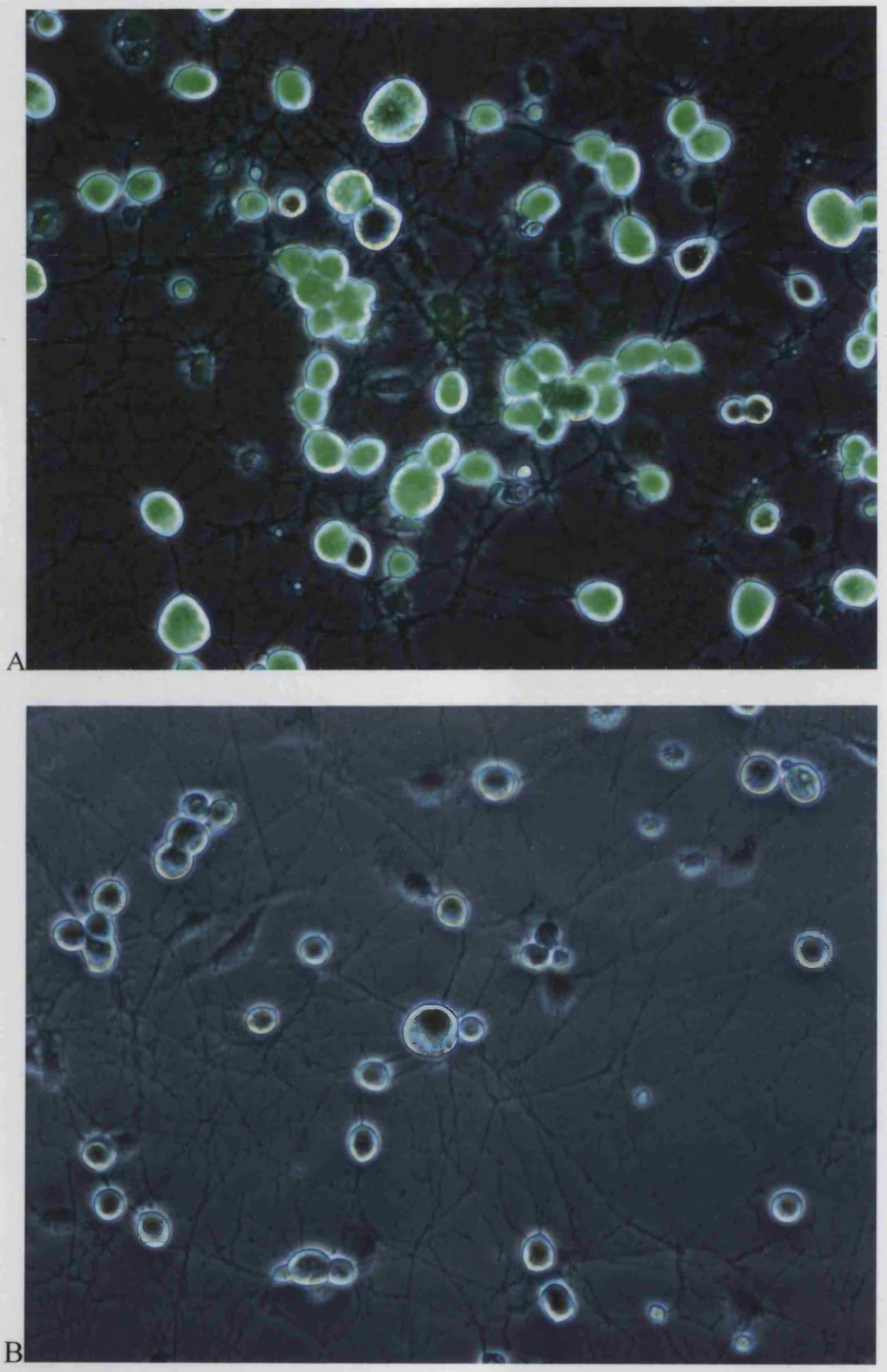


Figure 5.3: GFP expression in neonatal sensory neurons. A is a phase contrast view of the neonatal sensory neurons infected with the GFP virus, overlaid with the same field of view under fluorescent light. B is a phase contrast field of view of cells not infected with the GFP virus overlaid with the same field of view under fluorescent light, magnification for both pictures is x300.

5.4 The protective effect of heat shock proteins in neonatal sensory neurons

Having demonstrated that the virus vectors infect sensory neurons and express the correct proteins, the protective effect against lethal heat shock and hypoxic ischaemia was tested in neonatal sensory neurons (figures 5.4 and 5.5).

Figure 5.4 shows that HSP 27 and HSP 70 provided some protection to neonatal sensory neurons against heat shock, and figure 5.5 shows that HSP 27 and HSP 70 gave some protection against hypoxic ischaemia in neonatal sensory neurons. The HSV-1 virus vectors expressing heat shock proteins 27, 56 and 70 have previously been used in neonatal sensory neurons to test whether heat shock proteins can protect them from heat shock and ischaemia. As discussed in 5.1 HSP 27 and HSP 70 gave significant protection against heat shock and ischaemia in ND7 cells but HSP 56 although increasing mean survival slightly did not provide a statistically significant level of protection against either stress in ND7 cells. The results were the same in neonatal sensory neurons but the experiments were only repeated twice so statistical significance was not tested (Wagstaff, 1997).

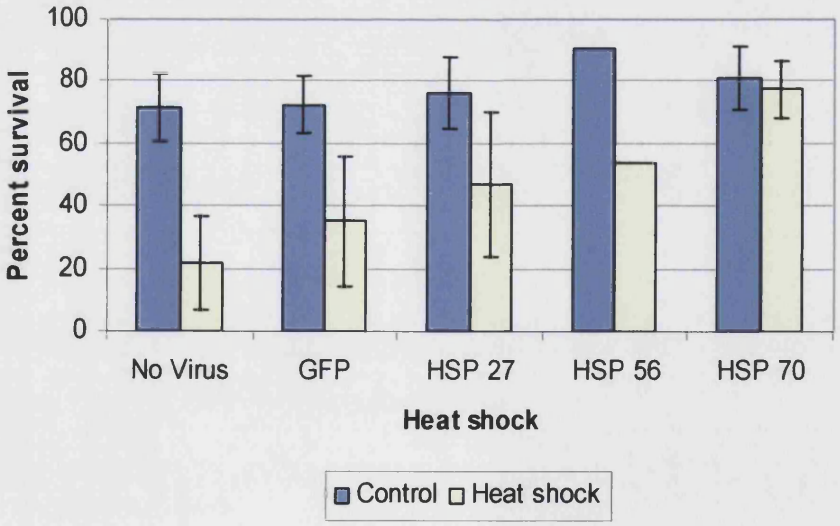


Figure 5.4: HSP overexpression from the viral vectors protects neonatal sensory neurons against heat shock. Heat shock was at 48°C for 10 mins. Survival was measured by morphology, n = 3 for all except HSP 56 where n = 1, error bars show the standard error of the mean.

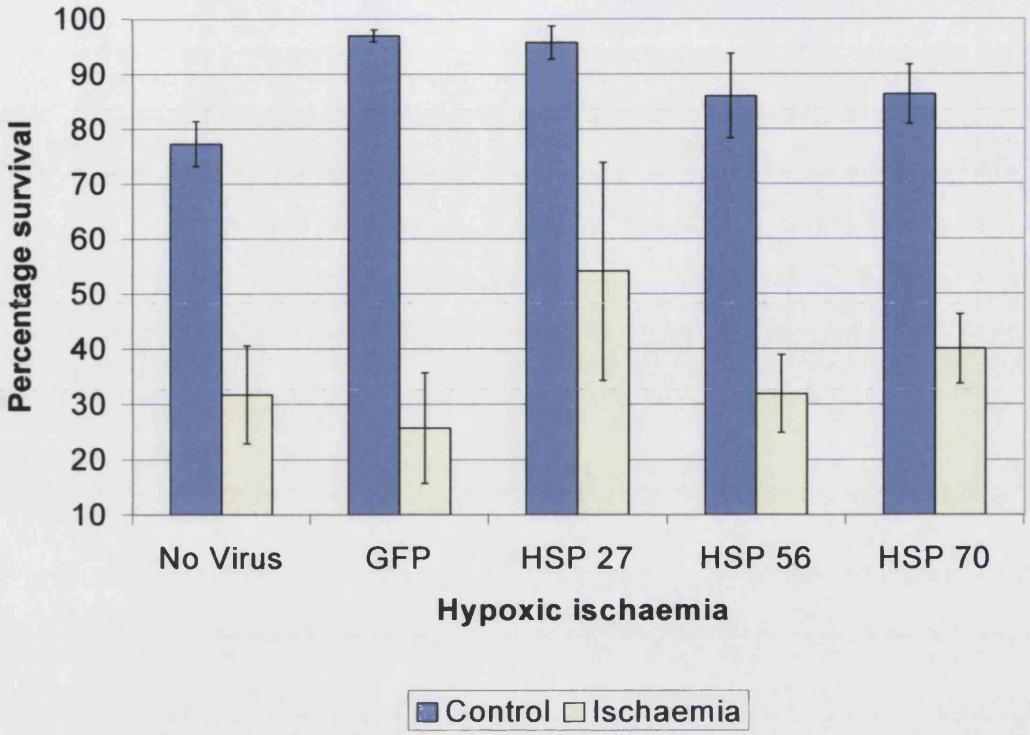


Figure 5.5: HSP 27 and HSP 70 protect neonatal sensory neurons against hypoxic ischaemia. The hypoxic ischaemic stress was a 3 hour stress, a 4 hour stress was used for all other experiments as described in the materials and methods. Survival was measured by morphology, n = 4, error bars show the standard error of the mean.

The experiments shown here were done to confirm the result and ensure that the new batches of virus acted the same way. HSP 56 appears to provide protection against heat shock in these experiments but this was only tested once and the results are otherwise the same. The trend shown in these results was that overexpressing HSP 27 or HSP 70 protects the neonatal sensory neurons against heat shock and hypoxic ischaemia. None of these results were statistically significant. As this work was confirmatory, the experiments were only repeated three times for heat shock and four times for hypoxic ischaemia, the variation in the level of death caused by the stress and the level of protection was quite large.

The aim of this chapter was to investigate whether aged neurons could be protected against stress by heat shock proteins. Having confirmed that the viruses infect sensory neurons and express the correct proteins, and having established the protocols for stressing the cells and assessing the survival and death of the cells afterwards, the viruses were used to infect sensory neurons from aged rats. The cells were exposed to heat shock or ischaemia, cell survival was measured by morphology and cell death by TUNEL as for previous experiments.

Figures 5.6 and 5.7 show the results of the experiments to test whether increased expression of the heat shock proteins in aged sensory neurons was protective against heat shock. In figure 5.6 the effect of the viruses was assayed by survival and in figure 5.7 TUNEL was used to assay the level of apoptosis.

5.5 The protective effect of heat shock proteins in aged sensory neurons

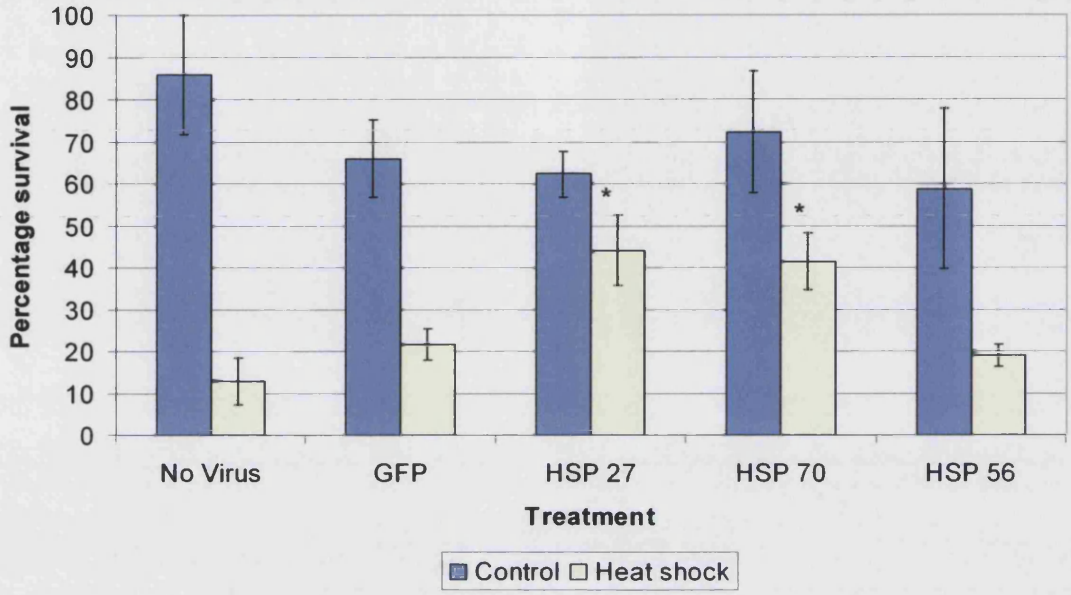


Figure 5.6: HSP 27 and HSP 70 protect aged sensory neurons against heat shock. Survival was measured by morphology. Heat shock was at 48°C for 10 mins, n = 3, *p = 0.05 and *p = 0.03 for HSP 27 and HSP 70 respectively after heat shock compared to GFP after heat shock, p = 0.8 for HSP 56 after heat shock compared to GFP after heat shock. The error bars represent standard error of the mean.

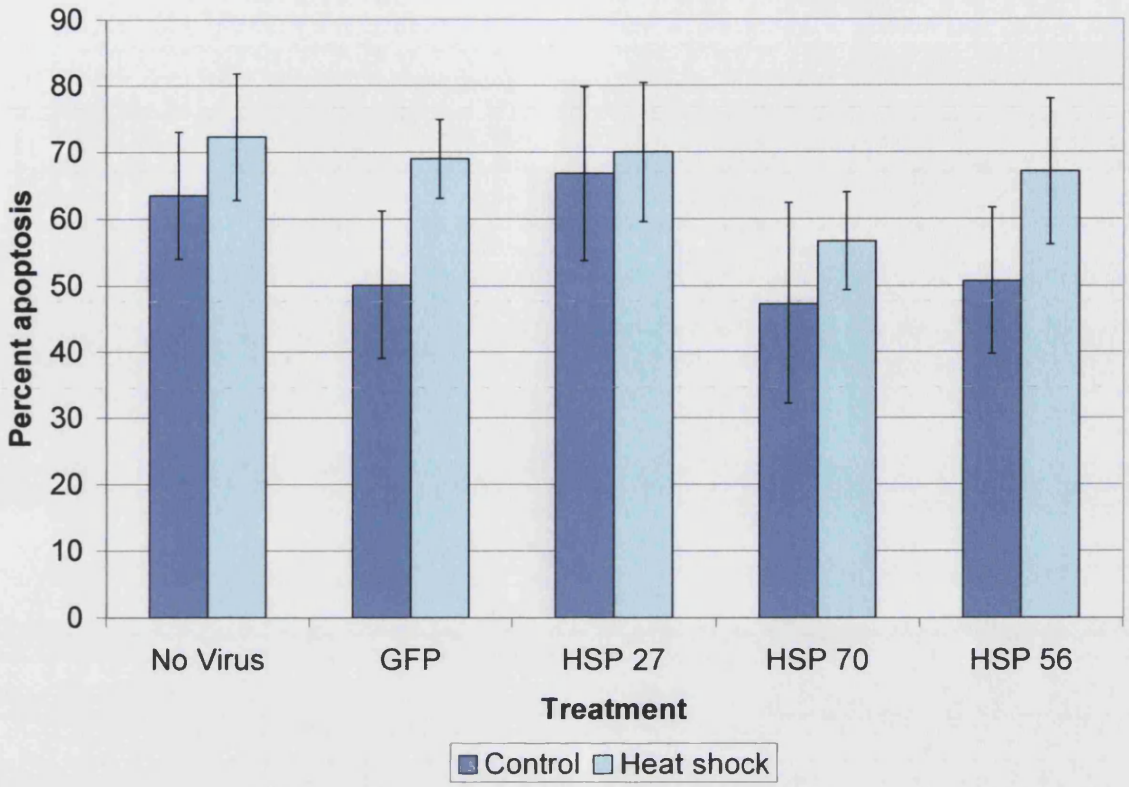


Figure 5.7: HSP 70 reduces death in aged sensory neurons exposed to heat shock, as measured by TUNEL. The TUNEL assay was carried out as described in the materials and methods. Heat shock was at 48°C for 10 mins, n = 4. The error bars represent the standard error of the mean.

Both HSP 27 and HSP 70 provided a statistically significant level of protection in the aged sensory neurons against heat shock in the survival experiment (figure 5.6), but only HSP 70 reduced the level of death in the TUNEL assay (figure 5.7). Importantly, this demonstrates that it is possible to use heat shock proteins to protect aged neurons against lethal heat shock.

The viruses were also used to test whether the aged neurons could be protected against lethal hypoxic ischaemia by heat shock proteins because ischaemic conditions such as stroke are much more common in older people than younger people. Figure 5.8 shows the results of the experiment designed to measure the effect of exogenous HSP expression on survival after hypoxic ischaemia in aged cells and figure 5.9 shows the results for the TUNEL assay to detect the effect of the viruses against ischaemia.

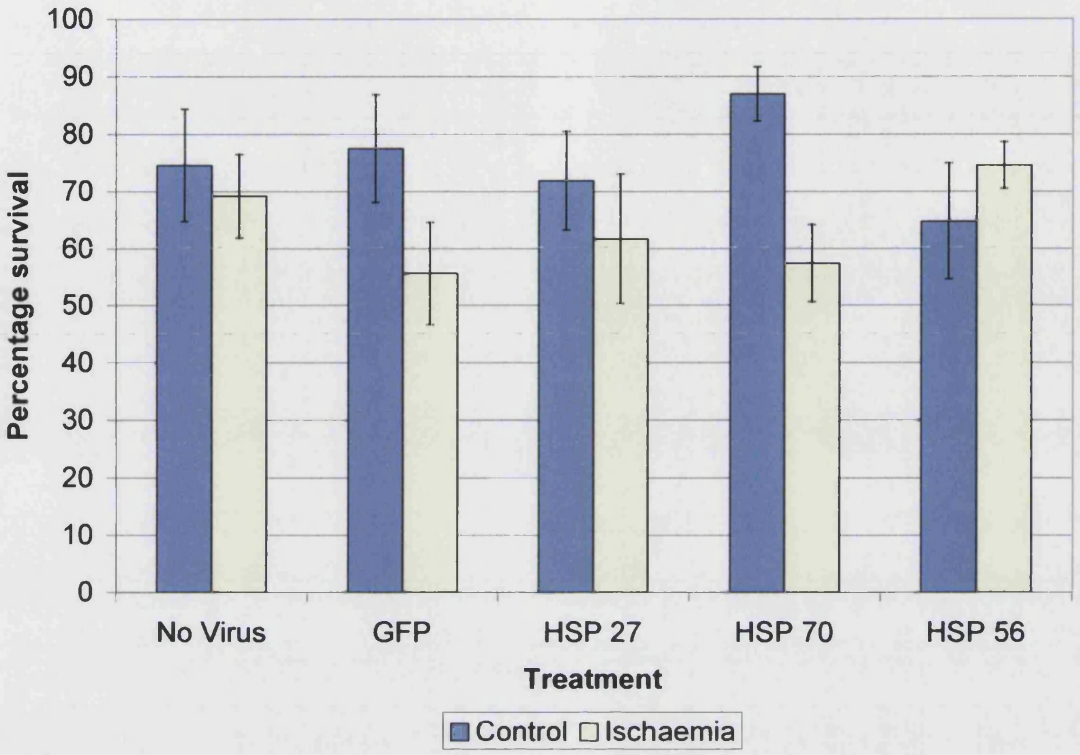


Figure 5.8: HSP 56 and HSP 27 reduce death in aged sensory neurons exposed to hypoxic ischaemia. The cells were subjected to hypoxic ischaemia for 4 hours and survival was measured by morphology, $p = 0.09$ when HSP 56 is compared to GFP, $n = 6$. The error bars represent the standard error of the mean.

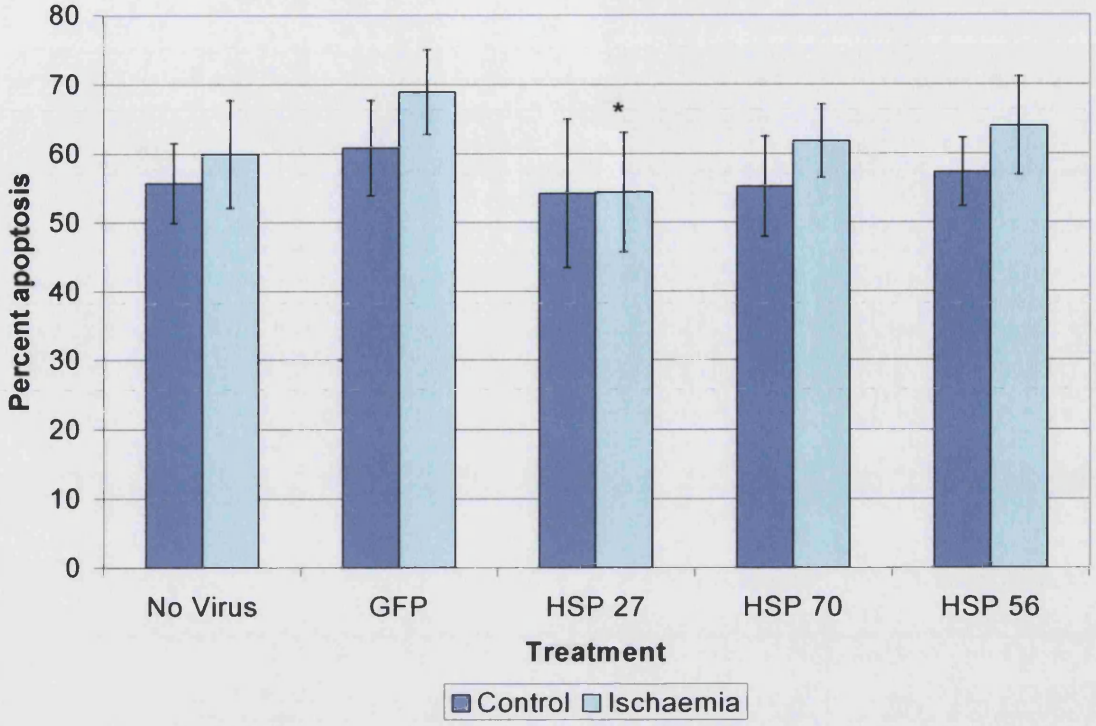


Figure 5.9: HSP 27 protects aged sensory neurons against hypoxic ischaemia, as measured by TUNEL. The cells were subjected to a 4 hour hypoxic ischaemic stress and the TUNEL assay was carried out as described in the materials and methods. HSP 27 after ischaemia was signifcant compared to GFP after ischaemia (* $p = 0.04$), $n = 5$. The error bars represent the standard error of the mean.

HSP 27 significantly reduced the level of apoptosis in aged sensory neurons after hypoxic ischaemia, compared to the GFP (figure 5.9). HSP 27 was able to increase survival of the aged sensory neurons after lethal hypoxic ischaemia slightly but this result was not statistically significant (figure 5.8). HSP 56 appeared to increase the survival of the aged cells after hypoxia, compared to the GFP, but this was not significant and it did not decrease the amount of death observed in the TUNEL assay after hypoxic ischaemia in aged cells (figure 5.9). Although HSP 70 reduced the level of death compared to the cells infected with the GFP virus it did not provide protection compared to the cells that were not infected (figure 5.9).

5.6 Discussion

This chapter has shown for the first time that heat shock proteins can protect aged sensory neurons from lethal heat shock and ischaemic stresses. It is particularly interesting that heat shock proteins can be overexpressed in sensory neurons because this suggests that the age related decline in the inducibility of heat shock proteins that has been described, see 1.3 and 5.1, can be overcome by artificial overexpression of these genes.

The results in neonatal sensory neurons presented in this chapter are similar to the results presented by Wagstaff (1997), with the two sets of experiments being performed by different individuals at different times using different batches of virus. These experiments were done as control experiments to ensure that the results in the neonatal cells were reproducible by different individuals and using different batches of the vector before beginning the experiments in the aged cells.

It is important to note that although the methods used for the hypoxic ischaemia were different, the results were the same. Wagstaff (1997) used ischaemic buffer and the plates were sealed to prevent gas exchange with the environment, whilst the hypoxic chamber was used in the experiments presented here because it creates a stress that is more similar to *in vivo* ischaemia, it not only exposed the cells to buffer with high lactic acid, low pH and no available sugar, it also gradually deprived the cells of oxygen. All three heat shock proteins HSP 27, HSP 56 and HSP 70 protected the neonatal cells against heat shock and both HSP 27 and HSP 70 protected the neonatal cells against hypoxic ischaemia. Wagstaff (1997) showed a small protective effect by HSP 56 against simulated ischaemia and a greater protective effect of HSP 70 against simulated ischaemia than is shown in this study, however these are small discrepancies and Wagstaff (1997) only performed the experiments twice.

Figures 5.1, 5.2 and 5.3 clearly showed that the virus vectors infected adult cells and that the infection resulted in a high level of expression of the relevant protein. Although western blots to show expression of the HSP genes from the viral vectors were not carried out in aged sensory neurons, due to limited amounts of material, the virus

vectors have been shown to be expressed in adult sensory neurons and ND7 cells. If the virus vectors were unable to infect aged sensory neurons or the cells were unable to express the transgene there would be no differences between the cells infected with different vectors.

In the neonatal cells the combination of the virus infection and the 4 hour hypoxic ischaemic stress was too great, there was 100% cell death, and the exposure to stress had to be reduced to a 3 hour period. Interestingly the susceptibility of the neonatal cells to heat shock was not affected by the infection with the virus vectors. In the aged cells the viruses appeared to have a slightly toxic effect, reducing both control survival and survival of the stressed cell compared to a smaller degree of death in the uninfected control and stress treated cells. However this was not observed in the neonatal cells (see figures 5.4 and 5.5 for the results of experiments in neonatal cells and figures 5.6 – 5.9 for the results of experiments in aged cells). In ND7 cells infection with the GFP and HSP 27 viruses induced a small increase in the level of HSP 70 suggesting that the infection of the cells with the virus vector was slightly stressful, therefore it would appear that the aged neurons were more susceptible to the stress of virus infection.

In the aged sensory neurons HSP 27 and HSP 70 gave significant protection against heat shock (figure 5.6). In the apoptosis assay only HSP 70 provided a protective effect (figure 5.7). Although HSP 27 did not provide a significant reduction in apoptosis after heat shock there was a higher level of apoptosis in the control cells infected with HSP 27 and surprisingly in the uninfected unstressed cells. It is possible that the HSP 27 virus had a greater toxic effect than the other viruses which was not revealed in the survival data Cells dying after infection with the virus vector but before the stress were excluded from the survival data by the first count of the number of cells alive before the stress. There does appear to be greater variability in the results of the apoptosis assay than the survival assay.

Surprisingly, HSP 56 was the only HSP to give substantial protection to the aged cells against hypoxic ischaemia compared to GFP (figure 5.8). HSP 27 gave a slight increase in the level of survival and gave a significant reduction ($p = 0.04$) in the level of apoptosis compared to GFP. HSP 56 did not decrease the level of apoptosis after

hypoxic ischaemia (figure 5.9). However, the maximum level of cell death in the survival assay was only 45% whereas in the apoptosis assay the maximum death was 70% and in both cases the GFP infected cells gave the highest level of cell death. If the survival had been measured at a later time point more death might have been revealed. In summary both HSP 27 and HSP 56 gave some protection against hypoxic ischaemia (figures 5.8 and 5.9).

Although providing some protection neither HSP 27 nor HSP 56 provided statistically significant protection from hypoxic ischaemia compared to uninfected cells. This suggests that the beneficial effects of expressing higher levels of heat shock proteins may be balanced against the damaging effects of infecting the cells with a virus vector. It also suggests that expressing higher levels of a single HSP does not completely restore the neurons to a young phenotype. It has been suggested that overexpression of individual HSPs although helpful in many situations may also be harmful. If levels of a single protein are too high it may interfer with normal cellular processes and cause chaperone sequestration and protein aggregation (Feder and Hofmann, 1999). Expressing higher levels of several heat shock proteins may prove to be more beneficial as may combining an increase in heat shock protein expression with an increase in proteasome activity perhaps by combining a constitutively active form of HSF-1 with a gene to express a proteasome subunit or the lamp2a gene to improve lysosomal degradation of damaged proteins (Cuervo and Dice, 2000; Chondrogianni et al., 2003).

In neonatal sensory neurons all three HSPs protected against heat shock and HSP 27 and HSP 70 protected against hypoxic ischaemia. In aged sensory neurons HSP 27 also protected against both stresses and HSP 70 protected against heat shock. The major difference between the neonatal and aged cells was HSP 56, which in aged cells did not protect against heat shock but surprisingly provided some protection against hypoxic ischaemia whilst HSP 70 did not, whereas in neonatal sensory neurons HSP 70 protected against hypoxic ischaemia and HSP 56 did not. Some of the results in aged cells were statistically significant but they were not in neonatal cells, despite the fact that both the neonatal heat shock experiment, (figure 5.4), and the aged heat shock experiment, (figure 5.6), were both repeated three times. The power of the statistical tests was low because of the low number of times the experiment was repeated, so

although the results were not statistically significant it is equally not possible to conclude that the results could have occurred by chance alone. Perhaps greater protection occurred in the aged cells because the stress could not induce the endogenous heat shock response whereas, the survival of the neonatal cells was not only dependent on the HSP expression provided by the viruses but was also influenced by the endogenous heat shock response. This could result in smaller differences in the survival/death between the uninfected and infected cells, and between the stressed and unstressed cells. However, the viruses clearly provided additional protection for the neonatal cells, demonstrating that increasing expression of HSPs is beneficial in all age groups.

The same virus vectors have also been tested by another member of the laboratory in peripheral blood mononuclear cells from young and aged people. Although the level of infection achieved was quite low, HSP 27 and HSF-1 protected both young and old cells against exposure to staurosporine and heat shock (Alsbury et al., 2004). In primary cardiac cells the HSP 27 and HSP 70 virus vectors gave protection against ceramide treatment and ischaemic stress (Brar et al., 1999b). Together with this thesis, this demonstrates that the HSP containing virus vectors may be useful in a variety of cell types against a variety of stresses in individuals of different ages.

Chapter 6: Discussion

This thesis sought to investigate potential ways to induce heat shock proteins in a non-harmful manner as a method of protecting neurons from lethal stress and to test whether aged neurons could be protected against lethal condtions.

Although CT-1 was protective in neonatal sensory neurons against hypoxic ischaemic stress (Chapter 3), attempts to use CT-1 and UCN to induce endogenous expression of the heat shock proteins were unsuccessful (Chapters 3 and 4). Levels of heat shock proteins that were not examined could have been affected as could the activity of the heat shock proteins, however this seems unlikely since the protective effect of CT-1 was limited to neonatal cells and UCN was not protective in sensory neurons of either age group tested.

CT-1 was used at a final concentration of 10ng/ml, this concentration was chosen for a number of reasons, in the work of Thier et al (1999a) 10ng/ml showed almost maximal results and high concentrations can cause erroneous results and so were avoided. If CT-1 is ever going to be useful as a treatment in vivo it needs to be effective at a low concentration to avoid side effects. Trials with CNTF in patients with ALS were problematic because at concentrations as low as 0.5, 2 and 5μg/kg/day the patients experienced numerous side effects including injection site reactions, cough, nausea, anorexia, weight loss and at the highest dose there was an increase in death most frequently caused by respiratory failure (Miller et al., 1996). When delivered at lower concentrations, 44 – 1230ng/ml, directly to the cerebral spinal fluid, other side effects were observed in some patients (Penn et al., 1997). No improvements in the disease symptoms were observed in either study although a longer term study may reveal medium and long term improvements. It is encouraging that the protective effect of CT-1 is observed at 10ng/ml but the results with CNTF which is a member of the same family caution us that the effect may not be significant enough to be useful therapeutically and the peptide may not be well tolerated. One potential solution would be to find a way to target CT-1 or other members of the family to neuronal cells, some work has been done to investigate this possibility (Bordet et al., 2001a).

The virus vectors were used as a direct method to exogenously express high levels of the heat shock proteins and to test whether aged sensory neurons could be protected against a lethal stress by heat shock proteins. Chapter 5 clearly shows that aged sensory neurons can be made to express high levels of HSPs and that the aged sensory neurons can be protected against stressful conditions by heat shock proteins. This suggests that manipulating heat shock protein expression could be useful clinically in treating diseases that affect elderly people.

One caveat of experiments done on primary neuronal cultures *in vitro* is the level of contaminating cells of other cell types. Centrifugation through a density cushion was used throughout this thesis to minimize the number of non-neuronal cells present in the final culture and in the cultures done in DMEM media the mitotic inhibitor AraC was used to prevent the growth and division of any contaminating non-neuronals. AraC was not used in combination with infection with the virus vectors as the combination resulted in some cell death. It was not used in conjunction with the F14 media because the non-neuronals do not thrive well in it, this is one of the advantages of using F14 media instead of DMEM, however, as well as the non-neuronals the neurons thrive better in DMEM which is why this was the main media used. Although low numbers of non-neuronal cells were present in the cultures the levels were the same in all treatments within experiments. Both the controls and the test cells were subject to the same levels of non-neuronals in the cultures so any effect of the non-neuronal cells was controlled for within each experiment and therefore cannot be responsible for the differences observed. However, differing levels of non-neuronal cells between experiments may explain some of the variability observed in the results. Since the cells for different experiments came from different individual rats, differences between the different individuals may also account for inter-experimental variability.

Caloric restriction is the only way currently known to delay ageing and extend life span and it has not been tested in humans. Caloric restriction was first found to affect lifespan in 1935 (McCay et al., 1935), this work was followed up and it was observed that caloric restriction robustly increases the lifespan of rats (McCay et al., 1939; Ross, 1961). This has also been shown in yeast, *C.elegans* and *D.melanogaster* (Klass, 1977; Chapman and Partridge, 1996; Jiang et al., 2000). There is some evidence that it may

retard the ageing process by increasing protein turnover and therefore reducing the burden of damaged proteins in postmitotic mouse cells (Lee et al., 1999). Interestingly in rats fed a calorie restricted diet, the decline in HSP 70 expression is reversed as is the observed decline in binding of HSF-1 to the HSE. After heat shock, in one study, only 16% of the aged rats fed *ad libitum* survived whereas 75% of the aged rats fed the restricted diet survived (Heydari et al., 1993).

Caloric restriction is obviously not a practical way to reduce the age related decline in health in humans, since it would be required throughout the lifetime of the individual particulary during the growing phase to be beneficial. There is no evidence yet that it would work in humans, and if it only delayed the effect of aging it would not be useful, we are not seeking to extend the lifespan of humans but to reduce the impact of diseases in the elderly. Hormesis would not be required throughout the lifetime of an individual to have some beneficial effects. Hormesis works by using a toxic stimulus at a dose low enough not to cause significant damage but high enough to induce heat shock protein expression (Neafsey, 1990), however, this relies on the preconditioning effect which we know is impaired in old individuals.

This thesis has shown that increasing heat shock protein expression artificially can protect aged cells against a subsequent lethal stress and therefore supports the idea that enhancing heat shock protein expression could be useful clinically in treating the elderly as well as the young.

It is undoubtedly useful to be able to increase expression of the heat shock proteins. Co-transfection of HSP 70 and HSP 40 either separately or together reduces aggregate formation in models of polyglutamine expansion neurodegenerative diseases (Chai et al., 1999; Kobayashi et al., 2000; Ohtsuka and Suzuki, 2000), and expression of HSP 70 also surpresses neurodegeneration in *Drosophila* models of a polyglutamine disease (Warrick et al., 1999; Auluck et al., 2002).

In time perhaps a way will be found to increase heat shock protein expression easily and without damage or risk to the patient so that the heat shock proteins can be used to protect individuals from harmful conditions such as surgery, chemotherapy or disease.

To achieve this either a safe and effective method of delivering exogenous genes or a safe means of inducing endogenous gene expression that works in aged cells would be needed. Some progress has been made in developing virus vectors. The HSP 27 containing virus vector used in this thesis was tested *in vivo* and protected hippocampal neurons against cell death caused by kainic acid induced seizures in rats (Kalwy et al., 2003). However, so far despite much research a safe and effective method of gene delivery in humans has not yet been found (Thomas et al., 2003). A retrovirus vector has been used to treat X-linked severe combined immunodeficiency disorder but due to the integration of the virus into the genome there was an unacceptably high incidence of cancer (Gaspar et al., 2003; Dave et al., 2004). The use of liposomes to treat cystic fibrosis, although initially promising, resulted in an immune response that rendered the treatment ineffective in the long term (Davies et al., 2001).

There are some candidates for inducing the endogenous expression of the heat shock proteins. Volloch et al (1998) have shown that the proteasome inhibitor MG132 can be used to restore endogenous HSP 70 expression. This presumably works by increasing the amount of damaged proteins accumulated in the cell to a level sufficiently high to induce heat shock protein expression. This could never provide a solution *in vivo* since inhibiting the proteasome will cause the cell to be overcome with damaged proteins and therefore senescence would rapidly occur (Chondrogianni et al., 2003). Another factor proposed to be capable of restoring stress resistance in aged cells is ascites HSP 72-inducing factor (AHIF) (Volloch and Rits, 1999). Little is known about this candidate at the moment and more work is needed to establish the identity and physiological functions of AHIF. One very promising candidate currently in development is bimoclomol, bimoclomol (N-2-hydroxy-3-(1-piperidinyl)propoxy]-3-pyridine-carboximidoyl chloride maleate) is a hydroxylamine derivative. Bimoclomol increases heat shock protein expression by prolonging the activation of HSF-1, it does this by prolonging the binding of HSF-1 to the HSE (Hargitai et al., 2003). Since it is the DNA binding activity of HSF-1 that becomes impaired with age it may be particularly useful in treating the elderly. Bimoclomol enhances HSP 70 expression in cell lines exposed to heat stress and improves wound healing in diabetic rats (Vigh et al., 1997). It has cardioprotective and antiarrhythmic effects (Jednakovits et al., 2000; Polakowski et al., 2002; Lubbers et al., 2002) and it reduces the symptoms of diabetic neuropathy and

retinopathy in diabetic animal models (Biro et al., 1997; Biro et al., 1998). What is particularly encouraging is that it has very little effect on heat shock protein production directly, under normal physiological conditions, it is only a coinducer of the HSPs which must be activated through the normal signalling pathways at the same time to work. This means that it does not affect healthy tissues and should have relatively few side effects. However, it has yet to be tested in aged cells.

On reflection the analysis and interpretation of the data presented in this thesis is problematic because the model is quite difficult to control. As a result of this there is considerable variability between different experiments and between the survival and death assays designed to measure the outcome of the same treatments. The control level of survival differed between different preparations of cells. The level of death between the stressed cells and the control cells for some experiments does not appear to be sufficiently large to be sure of detecting a protective effect.

The survival and death assays were very similar in design and therefore have the same basic flaws that they are dependent on the counting of individual cells by eye and involve sampling a population. It is also a problem that the two assays do not seem to complement each other but rather the two assays cast doubt on the validity of the other assay. It is also necessary to establish the type of cell death occurring, whether it is apoptotic or necrotic. An alternative apoptotic assay would have been useful to support the TUNEL assay since there was a concerning degree of variability in the results of the TUNEL assay. Annexin V staining was tried but was not included in this thesis because the experiment was designed in the same way as the TUNEL assay and the results were not very conclusive. A biochemical assay, such as the cytotox one assay from promega, would have been useful to examine necrotic cell death. The cytotox one assay, being a biochemical assay would complement the cell counting experiments, it is a membrane integrity assay that measures the amount of lactate dehydrogenase that has leaked out from damaged/dying cells into the supernatant. Since the results of this assay are linear compared to the total number of cells present the supernatant could be used to measure the level of lactate dehydrogenase, and the cells could then be used to provide a measure of the total protein and total DNA content. This would allow the lactate dehydrogenase

assay to be normalized by the total DNA and protein content to control for any differences in the total number of cells present.

In experimental science whether the data generated gives a positive or a negative answer is not the only thing to consider. Before investing heavily in a particular approach it is important to consider whether the experiment provides useful and useable information and whether the data reveals a change that is sufficiently large and robust enough to allow experimental manipulation. If it is not an alternative approach to the question should be tried rather than pursuing a set of experiments that ultimately will not increase our knowledge of the subject.

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